# Immune reconstitution in HIV-1 infection: the effects of antiretroviral and immune therapy

by

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## Declarations

*Originality*

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The following people and institutions contributed to the publication of the work undertaken as part of this thesis:

Paper 1

Restoration of HIV-1 specific responses in HIV-1 infected individuals changing from protease to non-nucleoside reverse transcriptase inhibitor containing antiretroviral therapy. *Scand J Immun* 2003; 57:600-7

Authors

Sullivan AK (40%- conception, recruitment, laboratory work, analysis and writing manuscript), Burton CT (laboratory), Nelson MR (recruitment), Moyle G

(recruitment), Mandalia S (data analysis), Gotch F (conception and manuscript review) and Gazzard BG (conception and manuscript review) (total 20%), Imami N (40% - conception, laboratory work, analysis and writing manuscript)

Paper 2

Interleukin-2 associated viral breakthroughs induce HIV-1-specific CD4 T-cell responses in patients on HAART. *AIDS* 2003; 17:628-9

Authors

Sullivan AK (45%-conception, recruitment and clinical management, laboratory work, analysis, manuscript), Hardy GAD (20%-conception, laboratory work), Nelson MR (patient recruitment), Gotch F and Gazzard BG (5%-conception, manuscript review), Imami N (30%-conception, laboratory work, analysis, manuscript)

Paper 3

A Randomised Trial of Subcutaneous Intermittent Interleukin-2 without Antiretroviral Therapy in HIV-Infected Patients: The UK-Vanguard Study. *PLoS Clin Trials* 2006; 1:e3

Authors

Youle M, Emery S, Fisher M, Nelson M, Tavel J, Fosdick L, Janossy G, Loveday C, Sullivan A, Davey R, Johnson M, Lane C. This was a large multi-centre trial. My role was the recruitment and clinical management of the patients at one of the three study centres, and review of the manuscript. However I conceived and managed the nested sub-study, performing all laboratory work and analysis (unpublished)

We the undersigned agree with the above stated "proportion of work undertaken" for each of the above published peer-reviewed manuscripts contributing to this thesis:

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| | |
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*Ethical conduct*

The research associated with this thesis abides by the International and United Kingdom codes on human and animal experimentation.

## Abstract

This thesis presents findings from two randomised, controlled pilot studies, with nested sub-studies, an observational study, a compassionate release programme and a mortality audit. It aims to examine the effects, at a clinical and cellular level, of antiretroviral and immunotherapy in HIV-1 infection. Combination antiretroviral therapy (cART) enables quantitative and a degree of qualitative immune recovery; however this is neither universal nor complete. The first part of the thesis explores the effects of cART on surrogate immune markers (SIM), treatment outcomes, disease progression and death. Significant variations are observed and further re-inforced by the mortality audit. I describe for the first time patterns of SIM decline and treatment response from which a model predicting treatment outcome could be developed. This section concludes with an observational study describing a differential effect on immune restoration of different classes of cART. Together this data suggests additional therapeutic interventions will be required to address the current inadequacies of cART to fully restore HIV-1 associated immune deficiencies. Therefore, the second part of the thesis examines the effect of interleukin-2 (IL-2) therapy in different settings; with and without cART, with therapeutic immunisation and in advanced disease. In the absence of cART, IL-2 increased CD4 T-cell counts without adversely affecting viral load or immune activation, potentially delaying the need for cART initiation. The compassionate release programme also demonstrated a 'delaying' effect which could be exploited in patients awaiting new therapies. The main therapeutic intervention study involved IL-2 and therapeutic immunisation in the context of cART, and reports novel findings of the acute effects of IL-2, including induction of HIV-1 specific responses. Overall increases were observed in CD4+, CD4+CD28+ and CD4+CD25+ T-cells, the latter being of particular interest as the precise

function of these cells in HIV-1 infection and following IL-2 therapy is still to be fully defined. As a pilot study these findings are preliminary but there is a trend for several effects to be more marked and more sustained in the arm combing all three treatments; suggesting therapeutic potential for combination immunisation and cytokine therapy which is worth pursuing. Despite the fact that the final IL-2 study in this thesis was completed in 2003, the recent publication of two large clinical outcome studies of IL-2, with somewhat unexpected results, makes these findings all the more pertinent today, and may afford some insight into the negative results observed in these large phase three trials. In summary, cART results in incomplete immune reconstitution, which can be enhanced by IL-2 and therapeutic immunisation at a cellular level. The challenge is to determine how, if at all, this can be translated into clinical benefit. Using additional SIM may help in targeting and monitoring therapy; the timing, scheduling and combination of which will be key and warrants further investigation.

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## Abbreviations

| | |
|---|---|
| AICD | Activation induced cell death |
| AIDS | Acquired immunodeficiency syndrome |
| APC | Antigen presenting cell |
| ARV | Antiretroviral |
| ARVT | Antiretroviral therapy |
| AUC | Area under the curve |
| bDNA | Branched DNA |
| CAND | Candida |
| cART | Combined antiretroviral therapy |
| CCR5 | Chemokine receptor 5 |
| CI | Confidence interval |
| CMI | Cell mediated immunity |
| CMV | Cytomegalovirus |
| CONA | Concanavalin A |
| cpm | counts per minute |
| CTL | Cytotoxic T lymphocyte |
| CVL | Community viral load |
| CWH | Chelsea and Westminster Hospital |
| DIR | Discordant immune response |
| DNA | Deoxyribonucleic acid |
| DTH | Delayed type hypersensitivity |
| DVR | Discordant virological response |
| EAP | Expanded access programme |
| EDTA | Ethylenediamine tetraacetic acid |
| FITC | Fuorescein isothocyanate |
| FLU | Influenza A |

| | |
|---|---|
| GALT | Gastrointestinal associated lymphoid tissue |
| HAR | High affinity receptor |
| Het | Heterosexual |
| HIV-1 | Human immunodeficiency virus type 1 |
| HPA | Health Protection Agency |
| HSV | Herpes simplex virus |
| HTL | Helper T-lymphocyte |
| IAR | Intermediate affinity receptor |
| ICL | Imperial College Laboratories |
| IDU | Intravenous drug user |
| IL-2 | Interleukin-2 |
| IQR | Inter-quartile range |
| IRC | Immune Response Corporation |
| IRIS | Immune restoration inflammatory syndrome |
| KS | Kaposi's Sarcoma |
| LAK | Lymphokine-activated killer cells |
| LAR | Low affinity receptor |
| LCA | Leukocyte common antigen |
| LPR | Lymphoproliferative response |
| LTFU | Lost to follow up |
| LTNP | Long term non-progressors |
| MAC | Mycobacterium avium complex |
| MFI | Mean fluorescence intensity |
| MHC | Major histocompatability complex |
| MIU | Million international units |
| MSM | Men who have sex with men |
| MTB | Mycobacterium tuberculosis |

| | |
|---|---|
| NK | Natural killer cells |
| NA | Nucleoside analogues |
| NHL | Non-Hodgkin's Lymphoma |
| NNRTI | Non-nucleoside analogues |
| OI | Opportunistic infections |
| PBMC | Peripheral Blood Mononuclear Cells |
| PBS | Phosphate buffered saline |
| PCL | Primary cerebral lymphoma |
| PCP | *Pneumocystis carinii* pneumonia |
| PCR | Polymerase chain reaction |
| PC5 | PE-cyanine 5 |
| PE | Phycoerythrin |
| PJP | *Pneumocystis jirove i* pneumonia |
| PHI | Primary HIV-1 infection |
| PI | Protease inhibitor |
| PPD | Purified protein derivative |
| PWM | Pokeweed mitogen |
| RNA | Ribonucleic acid |
| QOL | Quality of life |
| RCT | Randomised control trial |
| rIL-2 | Recombinant human interleukin-2 |
| SD | Standard deviation |
| SE | Standard error |
| SI | Stimulation index |
| sIL-2 | Soluble IL-2 receptors |
| SIM | Surrogate immune markers |
| STI | Structured treatment interruption |

| | |
|---|---|
| TCR | T cell receptor |
| TF | Treatment failure |
| TH1 | T helper type 1 |
| TH2 | T helper type 2 |
| TRECS | T-receptor excision circles |
| Tregs | Regulatory T cells |
| TS | Treatment success |
| TTOX | Tetanus toxoid |
| VL | Viral load |
| VLBD | Viral load below the level of detection |
| VZV | Varicella zoster virus |
| WB | Western Blot |

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## CHAPTER ONE: INTRODUCTION AND BACKGROUND

### 1.1 Human immunodeficiency virus type 1 (HIV-1) and the immune response

#### 1.1.1 Introduction and epidemiology

The Human immunodeficiency virus type 1 (HIV-1) causes immune dysregulation with hyperactivation and immune exhaustion leading to quantitative and qualitative immune deficiency. Without treatment HIV-1 infection leads to the acquired immunodeficiency syndrome (AIDS) and death. There have been significant advances over the past 30 years of the pandemic in the understanding of HIV-1 immunopathogensis and in developing effective treatments, in the form of combination antiretroviral therapy (cART), resulting in significant reductions in both the morbidity and mortality associated with HIV-1 (Pallela *et al.,* 1998, Lederman *et al*., 2000). Although treatment is becoming increasingly available in developing nations, the main treatment benefits to date have been seen in the developed world (UNAIDS). Understanding the mechanism of infection, the subsequent immune damage and the treatment associated reconstitution within the immune system, as well as identifying the persistent uncorrected immunodeficiencies should enable more effective and novel therapeutic interventions in those already infected, and may lead to potential preventative vaccination targets.

In the UK there are estimated to be 83,000 people living with HIV-1 (HPA, 2010). However up to one quarter are unaware of their status, and more than one quarter present with advanced disease (HPA, 2010). Those unaware of their status are therefore unable to access the therapeutic interventions they require and there remains a significant number of patients presenting with advanced immunological deficiency which may not be completely recoverable.

Two types of HIV infect humans - type 1 which accounts for the majority of infections world wide and type 2 which is seen primarily in western Africa and has a more indolent clinical course. HIV-1 is comprised of various clades and subtypes which have specific geographical distribution (Figure 1.1). Clade B predominates in the western world. Such viral variation is likely to have important implications for vaccine development.

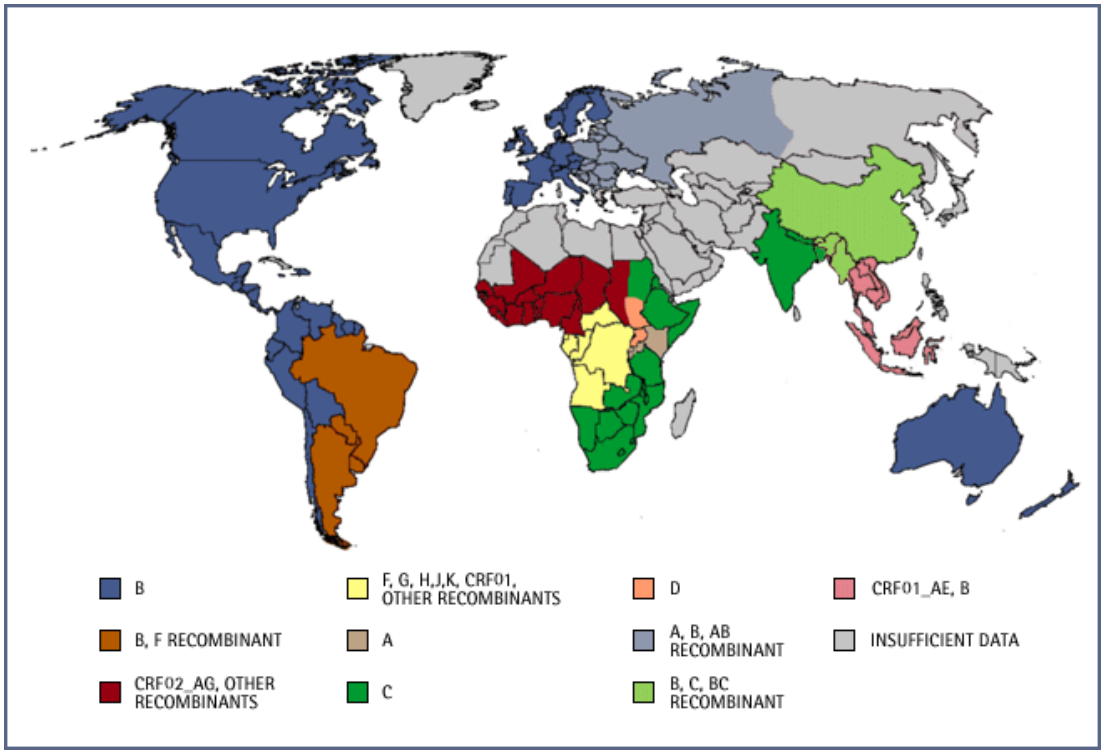



**Figure 1.1 Global distribution of HIV-1 clades (IAVI report, August 2003)**

### 1.1.2 Virology of HIV-1

HIV-1 is a retrovirus consisting of an outer envelope, a nucleocapsid layer, two identical strands of RNA, viral enzymes and proteins (Figure 1.2). There are 9 viral genes - the structural genes - *env*, *gag*, *pol*, the regulatory genes - *rev*, *nef*, *tat* and the accessory genes - *vpr*, *vpu*, *vif* (Subbramanian *et al*., 1994, Frankel *et al*., 1998, Seelamgari A *et al*., 2004).

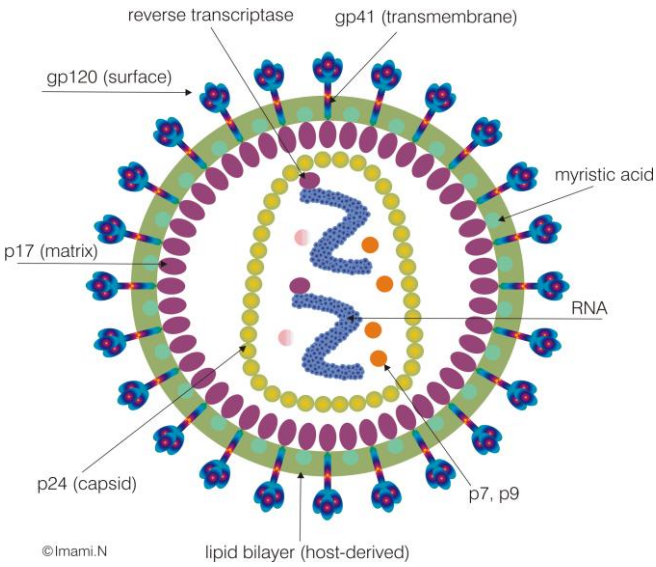



**Figure 1.2 Structure of HIV-1 virion**

HIV-1 can infect human T-cells, dendritic cells and macrophages. The understanding of the process whereby HIV-1 enters the cell has increased considerably in recent years. The virus enters its target cell by fusing its envelope with the cell membrane, the viral initiating glycoproteins responsible are gp120 and gp41 (Eckert *et al.*, 2001, Markovic *et al*., 2004). Initially thought to be the primary cell receptor for HIV-1, the CD4 receptor is now considered to play an accessory role to the chemokine receptors CCR5 (on dendritic cells, macrophages and T-cells) and CXCR4 (on activated T-cells) (Moore *et al.*, 1997, Alkhatib *et al*., 2007). These G protein coupled

receptors interact with gp 120 in the presence of CD4 and determine factors such as viral tropism; CCR5-R5 virus, macrophage-tropic and seen throughout infection and the lymphocyte-tropic CXCR4-X4 virus emerging with disease progression. Their ligands (CCR5: MIP-1$\alpha$, MIP-1$\beta$, RANTES and CXCR4: SDF-1) can block HIV-1 entry and mutations in the receptor genes can afford protection against infection (homozygous 32$\Delta$) or slow disease progression (heterozygous 32$\Delta$) (Blanpain *et al*., 2002). Viral tropism for these receptors now has implications for treatment with the recent development of receptor antagonsists such as maraviroc.

HIV-1 production rate is extremely high, although initially thought to be in the order of $10^9$–$10^{10}$ virions per day (Ho *et al.,* 1995, Perelson *et al*., 1996, Mohri *et al.*, 1998), subsequent work has downgraded this estimate to $10^3$ virions per day (Walker *et al.*, 1998, Rosenzweig *et al.*, 1998). The HIV-1 virus has a very high level of transcription errors which, when combined with its high replication rate, results in the daily production of multiple mutations, many of which are less fit than wild type or non-viable. Immune escape and subsequently, drug resistance tends to emerge in those patients on therapy (drug pressure) with incomplete viral suppression (Fischer *et al.,* 2010, Delaugerre, 2010).

### 1.1.3 Immune response to infection

This brief overview is aimed to highlight aspects of the response to infection particularly relevant to this thesis and is in no way meant to represent the entirety or complexity of the immune response.

Immature T lymphocytes, produced by the bone marrow, migrate to the thymus where positive and negative selection and T cell receptor (TCR) rearrangement result in the production of naive T-cells (CD45RA+CD62L+). A

by-product of this process is the production of T receptor excision circles (TRECS) and high levels within a cell are markers of recent thymic migration (Douek *et al.*, 1998). Exposure of the naive T-cell to antigen via antigen presenting cells (APC, *e.g*. dendritic cells) causes transformation to memory/effector T-cells (CD45RO+).

Infection causes an innate immune response followed by an adaptive immune response, which can be either predominantly type 1 or type 2. Type 1 (TH1) response is largely cell mediated immunity (CMI) and is the immune system's response to viral and other intracellular infections. CMI requires the interaction of APC, CD4 T-cells and CD8 T-cells and is further characterised by specificity and memory. Cytotoxic T-lymphocytes (CTL) kill the infected cell but there is evidence that in chronic viral infections this also requires virus-specific T-helper cells (Zajac *et al.*, 1998). Type 2 (TH2) response is predominantly an antibody response., also known as the humoral immune response. Both responses are associated with specific cytokine profiles; TH1 predominantly with IL-2, -12 and IFN$\gamma$ and TH2 with IL-4, -6 and -10. The development of one type of response tends to inhibit the development of the alternative response. This is of particular importance in the context of HIV-1 infection, where with disease progression TH2 response tends to dominante and thereby inhibits the more effective antiviral TH1 response (Clerici *et al.*, 1993).

Viral infections generate a CTL response which inhibits viral replication by direct killing and the release of soluble antiviral factors. Direct killing results from the infected cell releasing granzymes and perforin which, along with antiviral factors, are found in CTL secretory granules. This process results from the APC processing the viral antigen and, in the context of MHC class I, interacting with the CTL TCR (Yang *et al*., 1996, Wagner *et al*., 1998).

Additionally soluble antiviral factors are released. In HIV-1 infection these include β chemokines (eg. RANTES, MIP-1α and MIP-1β) which interact with the chemokine receptors and prevent further infection of CD4 T-cells. The phenomenon of class I restriction has important implications for an individual's ability to mount an effective immune response as it determines the viral peptides presented to the TCR. This impacts on the possibilities for immune manipulation and may well limit its potential as a sustainable therapeutic intervention. In addition to CTL there is generation of virus specific T-helper cells which occurs following antigen presentation by the APC in the context of MHC class II. These T-helper cell responses are essential for the maintenance of CTL. T-help probably consists of a combination of direct cell-cell interaction and the release of cytokines such as IL-2 (Smith *et al.*, 2008).

### 1.1.4 T-Lymphocyte surface receptors

All these immunological processes occur subsequent to various cell surface receptor/molecule interactions. The presence of specific receptors or molecules on a cell surface identifies a cell's functions, capabilities and to a certain extent history and previous contacts (Appay *et al.*, 2008). There are numerous surface receptors with a wide variety of functions. Described below are those relevant to this thesis.

*1.1.4.1 CD3*

The CD3 molecule is expressed on mature T-lymphocytes and some thymocytes. This 16-25 kDa molecule consists of 5 polypeptide chains and is an integral part of the TCR complex. It participates in antigen recognition (MHC I and II bound peptides) and T-cell activation (van Agthoven *et al.*, 1981). Normal ranges quoted vary from approximately 65-78% of peripheral

blood mononuclear cells (PBMC) being CD3 positive (CD3+) to 55-84 % (Bisset *et al*., 2004). In HIV-1 infection signalling across the TCR/CD3 receptor may be affected by the virus (Iafrate *et al.,* 1997).

The presence of CD3 receptors on cells was used in the phenotypic analyses detailed in this thesis to enhance identification of the T-cell population for further analysis.

*1.1.4.2 CD4*

The CD4 molecule is present on T-cells (approximately 45% of PBMC), thymocytes (80%, co-expressed with CD8 on immature cells) (Micelli *et al*., 1991), eosinophils, dendritic cells and macrophages (100%, but at a lower density compared to T-cells) (Hannet *et al.*, 1992). This 59 kDa transmembrane glycoprotein is a member of the Ig superfamily. It has an extracellular domain, which with the TCR complex interacts with the MHC class II molecule on the APC (Sakihama *et al.,* 1995), enabling antigen recognition and subsequent T-cell activation (Smith-Garvin *et al.*, 2009). Its intracellular domain causes the activation of the protein tyrosine kinase Lck, initiating transcription and cellular activation (Ryan *et al*., 1995). The normal range of CD4+ T-cells in the peripheral blood of an uninfected individual is 450-600 cells/µl, 35 - 45% (Imperial College Laboratories (ICL), London 2010), with different ranges given by other authors; 309 -1139 cells/µL, 32.5 – 63.0% (Bisset *et al*., 2004) and 715–1085 cells/ µL (Autran *et al*., 1997).

The CD4 molecule is a receptor for the gp120 molecule of HIV-1, binding it with high affinity (Berger *et al.,* 1999), with subsequent down regulation of CD4 expression. MHC-class II dependent binding requires CD4 tetramerisation, whereas only single CD4 molecules are required for gp120 binding. Additionally, *nef* has been shown to induce endocytosis and degradation of CD4 in lysosomes and to disrupt the association of CD4 with

Lck (Anderson *et al.,* 1993, Rhee *et al.,* 1994, Aiken *et al,* 1994, Salghetti *et al.,* 1995, Iafrate *et al.,* 1997). There is low level expression of CD4 on some mature, activated CD8+ T-cells peripherally which may explain CD8 T-cell infection by HIV-1 (Kitchen *et al.,* 1998). CCR5 interaction only requires low density CD4 expression, such as is found on macrophages, however CXCR4 requires high density, as seen on activated T-cells, and this may explain some of the viral tropism seen in HIV-1 disease.

In HIV-1 infection the number and percentage of CD4+ T-cells decrease with time. In the majority of patients effective cART results in an increase in CD4 T-cells often to within the normal range. Additionally exogenous IL-2 increases the number of CD4 T-cells.

*1.1.4.3 CD8*

The CD8 molecule is present on CTL and some NK cells, but at a lower density (Terry *et al.,* 1990). This molecule consists of an $\alpha$ and a $\beta$ chain, 32-34 kDa each. The extracellular domain of the CD8 receptor interacts with the MHC class I molecule on APC (Salter *et al.,* 1990). Its intracellular domain also interacts with the protein tyrosine kinase Lck (Anderson *et al.*, 1993), initiating cellular activation.

The normal range of CD8+ lymphocytes is 190-1210 cells/µl (15-50%) (ICL), with other reference ranges being 137 – 823 cells/µL, 11.6-38.6% (Bisset *et al*., 2004) and 385-715 cells/µL (Autran *et al*., 1997). The number of CD8+ T-cells increases in response to any viral infection, including HIV-1. HIV-1 infection has additionally been shown to down-regulate the expression of CD8 on infected cells (Appay *et al.*, 2000). The number of CD8 T-cells also declines with disease progression, albeit at a later stage compared to the decline in the CD4 T-cell count, and the initiation of cART tends to return the ratio of these cells towards normal.

*1.1.4.4 Naive and memory T-cell surface markers – 45RA, 45RO and 62L*

The CD45 molecule, previously known as the leukocyte common antigen (LCA) is expressed as isoforms with different molecular weights. CD45RA is the 220 kDa isoform, incorporating exons A,B and C, and is expressed on a subset of T-cells, B cells and monocytes. The 180 kDa isoform, lacking these exons, is CD45RO, found on 70% CD4+ T-cells, 35% CD8+ T-cells, thymocytes and monocytes (Janeway, 1992, Wills *et al.*, 1999). There is a sub-population of T-cells which co-express both CD45RA and CD45RO (Zola *et al*., 1992, Prince *et al*., 1992). CD45 regulates T-cell responses to antigens, influencing activation (De Jong *et al*., 1991). CD45RA T-cells are also referred to as naïve cells, and CD45RO T-cells as memory or effector cells (Wills *et al*., 1999) When CD45RA+ T-cells interact with an antigen they become activated, express CD45RO and down regulate CD45RA. These CD45RO+ cells can subsequently respond to recall antigens. CD45RO facilitates CD4 molecule grouping on the T-cell surface, enabling interaction with APC (Janeway, 1992), as this requires CD4 tetramerisation. The proportion of CD45RO+ cells increases with age (Wills *et al.*, 1999). Reversion from CD45RO+ to CD45RA+ has been documented within both the CD8+ and CD4 T-cell populations (Appay *et al.*, 2008).

In HIV-1 infection the CD45RO T-cell population declines throughout the course of the disease. This is reflected in the gradual loss of response to recall antigens and mitogens as would be expected with ongoing loss of effector cells. It correlates well with the observed clinical course. With disease progression and loss of these effector cells individuals become increasingly susceptible to various opportunistic infections, including those to which they had previously mounted an adequate immune response.

cART results in an early rapid increase in CD45RO+ T-cells, a significant proportion of which is probably due to redistribution. This is followed by an increase in CD45RA cells in which thymic contribution probably plays a role (Autran *et al.*, 1997, Pakker *et al.,* 1998), along with peripheral expansion and decreased apoptosis. This is reflected clinically in improved protection against opportunistic infections, permitting the cessation of primary and secondary prophylaxis (El-Sadr *et al.*, 2000), and the occurrence of immune reconstitution inflammatory syndrome (IRIS) (Zegans *et al.*, 1998, Shelburne *et al.,* 2003, Antonelli *et al*., 2010).

CD62L is an adhesion molecule expressed on the surface of T and B lymphocytes, which can be used in addition to CD45RA to identify the naïve cell pool, as it is expressed on unstimulated cells (Hannet *et al.,* 1992) and interaction with antigen causes down regulation (Springer *et al.,* 1994). 62L binds to the high endothelial venules in the lymph nodes, allowing naive cell recirculation (Bevilacqua *et al.*, 1993).

Quoted normal ranges for naïve and memory CD4+ and CD8+ T-cells (Bisset *et al*., 2004);

| | | | |
|---|---|---|---|
| Naïve | CD4/CD45RA/62L+ | 84-761 cells/μL | 9.5–41.9% |
| | CD8/CD45RA/62L+ | 42–360 cells/μL | 3.7–19.2% |
| Memory | CD4/CD45RO+ | 247–807 cells/μL | 16.5–42.2% |
| | CD8/CD45RO+ | 72–377 cells/μL | 3.8–22.8% |

*1.1.4.5 Activation markers – HLA-DR and CD38*

HLA-DR (class II, 29-33 kDa) is expressed on B-cells, activated T-cells (Hannet *et al.,* 1992) and monocytes. It is involved in the interaction between lymphocytes and macrophages, T and B cells and in cytotoxicity (Sonderstrup *et al.,* 1998).

The hyperactivation of the immune system associated with HIV-1 infection results in a multitude of changes, one of which is an increase in HLA-DR expression (Douek *et al.*, 2009). CD8+HLA-DR+ cells have decreased proliferative capacity (Pantaleo *et al.*, 1990) and the increased levels are associated with T:T presentation leading to a state of anergy (Lombardi *et al*, 1997). Initiation of cART with subsequent control of viraemia leads to down regulation of HLA-DR.

The normal range of CD4/HLA-DR+ T-cells is 11-55 cells/μL, 0.8-4.4%, and CD8/HLA-DR is 6-108 cells/μL, 0.3-6.4% (Bisset *et al*., 2004).

The CD38 molecule is 45 kDa and is expressed on the surface of activated T-cells and plasma cells. It is involved in the activation and regulation of T and NK cells (Funaro *et al.,* 1990). As with HLA-DR, high levels of expression reflect hyperactivation of the immune system as occurs in HIV-1 infection. It has been identified as a surrogate marker of disease progression. Initiation of cART results in decreased expression on T-cells.

The normal range of CD4/CD38+ T-cells is 69-547 cells/μL, 6.1-32.2%, and CD8/CD38+ is 13-124 cells/μL, 0.9-7.0% (Bisset *et al*., 2004).

*1.1.4.6 Co-receptor CD28*

CD28, a 44 kDa molecule, is expressed on the majority of CD4 T-cells and on 50% of CD8 T-cells (Lenschow *et al.,* 1996). It binds with CD80 and CD86 on APC (*Wang et al.,* 2004, Corthay, 2006), providing the essential signal 2 in T-cell activation. This stabilises the mRNA for IL-2 transcriptional factors, enhances mRNA production and increases cell surface IL-2 receptor expression (Barker *et al.,* 1997). This co-stimulatory signal plays a role in T-cell differentiation and proliferation (Lenschow *et al.,* 1996), maintenance of peripheral tolerance (Schwartz *et al.*, 1996, Chambers *et al.*, 1997) and the prevention of apoptosis. Its deficiency results in T-cell anergy.

There is progressive loss of CD28 expression on T-cells in HIV-1 infection, being most marked in the CD8+ population. This down regulation correlates with disease progression, decline in CD8+ T-cell antiviral activity (Landay *et al.*, 1993, Barker *et al*., 1997, Imami *et al.,* 1999), and increased viral replication (Haffar *et al.,* 1995).

*1.1.4.7 IL-2 receptors*

Interleukin-2 receptors are discussed in section three of the Introduction.

### 1.1.5 Immune response to HIV-1 infection

*1.1.5.1 Cell mediated immune response to HIV infection*

HIV-1 infection results in both quantitative and qualitative immune dysfunction, characterised by hyper-activation and immune exhaustion (Gea-Banacloche *et al.*, 1999). Understanding the immunopathology of HIV-1 infection and the effects (beneficial and partial) of cART on the immune system will guide identification of potential targets for immune therapy. It would appear with our current therapies viral eradication is not yet possible, largely due to viral latency in long term effector cells, and the next therapeutic breakthrough may be through manipulation of the immune system. The immune system plays a key role in determining the viral set point and disease progression, and examination of immune parameters in different clinical cohorts in regards to disease progression can also inform these potential strategies.

In primary HIV-1 infection (PHI) HIV-specific CD8 CTL and CD4 T-helper lymphocytes (HTL) are produced which initially control viral replication and determine the viral set point (Koup *et al*., 1994, Borrow *et al*., 1997, Goulder *et al*., 1997, Price *et al*., 1997, Kent *et al*., 1997). Depletion of CTL prior to SIV infection in macaques causes loss of this initial viral control (Matano *et al*.,

1998, Jin *et al*., 1999, Schmitz *et al*., 1999). The high replicative and mutational rate of HIV-1 results, over time, in viral evolution and the emergence of viral swarms and quasi species able to evade the immune response. Studies suggest that CTL are subsequently deleted in the presence of the resulting persistent high viral load (Moskophidis *et al.*, 1993). This deletion may also represent a qualitative deficiency as well as a quantitative reduction, with persistence of some dysfunctional or anergic cells. (Goulder *et al.*, 1999). The activated CD4 T-cells are prey to this elevated viral load (Schnittman *et al.*, 1990) and increased rates of infection, dysfunction and destruction result, with a degree of HTL dysregulation also occurring prior to the actual decline in numbers of CD4 T-cells. It is this loss of strong HIV-1 specific helper T-cell responses that is the most marked deficiency in advanced disease (Miedema *et al.*, 1994). This loss of HTL function would impair events downstream, including activation of CTL. Another mechanism may be cells undergoing activation-induced cell death in the presence of an high antigen load (Abbas *et al.*, 1996). Therefore with early intervention in PHI we see enhanced HIV-1 specific T-helper cell function (Rosenberg *et al.*, 1997, 1998, Al-Harthi *et al*., 2000, Oxenius *et al.*, 2000, Malhotra *et al.*, 2000, Deeks *et al.*, 2000). If cART is commenced in chronic infection reduced HIV-specific immunity, especially CTL, is observed (Kalams *et al.*, 1999, Ogg *et al.*, 1999, Markowitz *et al.*, 1999). This is presumably due to lack of ongoing antigenic stimulation required to maintain these responses, and this loss may be accelerated by lack of T-help. This qualitative immune deficiency may also be partly related to IL-2 dysregulation and activation induced anergy. During HIV-1 infection a reduction in the production of IL-2 is observed. This has been shown to occur in the early stages of infection with production becoming more limited during progression of the disease (Gruters *et al.*,

1990, Shearer *et al.,* 1992, Barker *et al.,* 1997). It is likely this phenomenon is related to a switch from a TH1 to a TH2 immune response, in which the predominant cytokines change from IL-2 to IL-4 and IL-10 which in turn further inhibit IL-2 production (Clerici *et al.*, 1994, Barker *et al*., 1997).

Furthermore, early in HIV-1 infection there is migration of virus to the gastrointestinal associated lymphoid tissue (GALT) with subsequent infection and marked loss of CD4 T-cells (Brenchley *et al*., 2004, 2006). This allows translocation of microbial antigens, resulting in generalised immune system activation (Brenchley *et al*., 2006). This persists in chronic infection as the loss of GALT associated CD4 T-cells is not restored by cART, and thus the associated immune system activation remains uncorrected.

The resulting chronic immune system activation is reflected in high levels of CD4 and CD8 T-cell activation markers (*e.g*. CD38, HLA-DR) seen in chronic HIV-1 infection (Liu *et al*., 1997), which correct towards those levels seen in HIV-1 uninfected individuals but do not completely normalise on cART initiation.

The ability of an individual to mount an effective immune response to HV-1 is likely to be genetically determined to a significant extent (Hill, 1998) and therefore has significant bearing on the prospect of sustainable therapeutic immune system manipulation. This has important clinical ramifications as viral load is a primary determinant of disease progression and, as early as six months post PHI, is highly predictive of subsequent disease progression (Lyles *et al.*, 2000). The resultant level of immune system activation is as important, if not more so, in predicting disease outcome (Liu *et al*., 1997, Douek *et al.*, 2009). Despite a degree of initial HIV-1 control however, the majority of individuals experience progressive immune dysfunction with resultant immunodeficiency, progressing to AIDS and death. With chronic infection,

the ongoing high viral replication and associated immune activation results in immune exhaustion. The subsequent increase in viral replication is due to both viral and host factors.

In many other viral infections the immune system mounts an effective protective response, sufficient to contain, but not eradicate the virus. However this does not occur in the majority of patients infected by HIV-1. There is a small group of individuals who are able to control the virus and protect their immune system for prolonged periods of time (Deeks *et al*., 2007). These individuals, known as long term non-progressors (LTNP) are an important source of data regarding those factors critical to viral control. LTNP are defined as individuals infected by HIV-1 for at least 10 years, with a CD4 T-cell count >500 cells/μL and a viral load <1000 copies/ml in the absence of antiretroviral therapy. LTNP account for <1% of individuals infected with HIV-1 but reports indicate that all individuals will eventually progress (Westrop *et al*., 2009, Blankson, 2010). It is likely that the partially protective immune response these individual mount is genetically determined. Immunologically these individuals have broad, strong virus specific CTLs (Harrer *et al.*, 1996, Gea Banacloche *et al.*, 2000), strong virus specific HTLs (Rosenberg *et al.*, 1997, 2000), high levels of chemokines, low levels of activation markers and, in some cases, neutralising antibodies. In some LTNP strong virus specific CTL responses have been identified 20 years post PHI. In effect the LTNP immune response is characterised not only by its strength but also by its breadth, which is likely to be highly genetically determined (Valdez *et al*., 2002). The breadth of CTL and HTL better enables the patient to deal with the increasingly diverse HIV viral swarms. LTNP in some cases have shown the presence of a strong response to *env* and *gag* proteins (Riviere *et al.*, 1995, Yang *et al*., 1996, Rosenberg *et al.*, 1997, 2000, Pontesilli *et al*., 1998, Ogg *et*

*al*., 1998, Oxenius *et al.*, 2000, Malhotra *et al.*, 2000) with an associated negative correlation with viral load (Kalams *et al.*, 1999). LTNP have higher levels of β chemokines (Gruters *et al.,* 1990, O'Brien *et al.*, 1998) which have a direct antiviral effect and will protect against cellular infection to a degree. Delineating LTNPs' immune system characteristics has identified possible therapeutic goals for immune manipulation. The vital role of an advantageous genetic profile somewhat dampens the optimism regarding these as targets for immunotherapy with sustainable outcomes.

Other host genetic factors such as polymorphisms in chemokine co-receptors are associated with different rates of disease progression (Smith *et al.*, 1997, Martin *et al.*, 1998, O'Brien *et al.*, 1998). HLA alleles also have associations with the rate of disease progression, which is likely to be related to the ability of the host to present different viral epitopes.

There are also viral-determined factors which influence disease progression and potentially affect both immune and treatment responses. With progression of disease, viral phenotype switches from non syncitial inducing (NSI) to syncitial inducing (SI) and from CCR5 to CXCR4, with implications for tropism and fitness. Other viral related factors include the *nef* induced down-regulation of HLA class I expression (Schwartz *et al.*, 1996, Le Gall *et al.*, 1998) reducing the effective immune presentation of antigen.

The existence of viral reservoirs is another important factor in the persistence of disease and the present pessimism regarding eradication. Soon after HIV-1 infection a population of CD4 T-cells that harbour HIV-1 in a transcriptionally silent state are generated (Pierson *et al.*, 2000). The virus in these cells is in a post integration latent state and therefore not susceptible to immune recognition. Activation of these cells would expose them to attack but would also allow them to seed other susceptible cells, re-establishing the reservoir.

Several recent studies of IL-2 therapy have addressed the possibility of flushing these reservoirs but this approach has not been shown to be effective to date. Recent drug developments address this viral latency with a view to eradication. It is now recognised these cells are the more likely source of low level viral blips observed clinically, rather than on-going low level viral replication (Kelleher, 2008).

The natural history of HIV-1 infection is therefore one of increasing viral load and diversity causing immune system hyperactivation and thus decreasing T-cell number and function, both CD4 and CD8, through direct killing, activation induced cell death and anergy.

*1.1.5.2 Humoral immune response to HIV infection*

The other arm of the adaptive immune response to HIV, referred to as the humoral response, results in the generation of HIV specific antibodies (Ab). There are several main Ab categories or processes; binding antibodies (bAb), neutralising antibodies (nAb), antibody-dependent cellular cytotoxicity (ADCC) and complement binding antibody.

Antibodies are immunoglobulins produced by B cells and can be innate or antigen specific. The humoral response is closely interlinked and dependent upon cell mediated immunity; CD4 T-cells are integral to this via essential helper/inducer function; achieved by cell to cell contact and local release of regulator cytokines (*e.g*. IL-2, IFN-gamma). Antibodies are key components in host defences. They are seen as being of increasing importance in HIV vaccine research and development, given the lack of success to date of vaccines capable of inducing either therapeutic or protective cell mediated immunity to HIV.

HIV Ab are produced within 1-2 weeks of acute infection; they circulate in the blood and are secreted at mucosal surfaces. The kind of activity an

antibody has depends on its isotype; light chain specificity and heavy chain class, and this can also be influenced by the cytokine milieu. The specificity of anti-HIV antibodies evolves over time with *gag* responses appearing first followed sequentially by *nef*, *rev* and finally *env*, and a stable peripheral B cell repertoire is established over the first year of infection. IgG appears first and is followed by the production of IgA. Following HIV infection B cell dysfunction is seen with polyclonal activation, hypergammagloulinaemia and impaired primary and secondary responses (innate and adaptive).

*Binding Antibodies*

Binding antibodies (bAbs) may or may not neutralise HIV infectivity but could potentially be protective as suggested by the finding of a degree of protection in a recent Thai vaccine trial (Rerks-Ngarm *et al*., 2009) where high levels of bAb were seen in vaccinated volunteers, and fewer vaccinated individuals had nAb compared to bAb.

*Neutralising Antibodies*

B cells produce specific Ab in response to HIV which bind to the virus and neutralise it. The neutralising Ab (nAb), usually IgG, are specific for the HIV envelope glycoproteins gp120 and gp41, particularly the CD4 binding region and co-receptor binding site of gp120. Those Ab directed against gp120 tend to be clade specific, whereas gp41 Ab tend to have wider interclade activity. There are both conformational and continuous determinants within envelope, and the response appears to evolve over time. nAb are often only specific for the initial native virus and the loss of cross or broad reactivity is now thought to be more closely related to conformational change in gp120 following sequential binding to CD4 and co-receptors. High antibody titres are required for neutralisation as any protein left unbound can bind to target cells and initiate fusion and thence infection. High levels of nAbs against

common laboratory strains are frequently encountered, however levels of nAb directed against an individual's own virus are less consistent reflecting a loss of effective reactivity. nAb are able to prevent infection of susceptible cells but are ineffective once infection has occurred.

*Antibody Dependent Cellular Cytotoxicity (ADCC or enhancing Antibodies)*

Antibodies to viral *env* can also induce ADCC which is mediated by NK cells and macrophages. HIV specific Ab (IgG1) bind to infected cells which express the viral epitopes gp120 and gp41 on their surface. The Fc region of this bound Ab then binds to NK cells or macrophages via IgGFc or complement receptors, and induces ADCC. Two mechanisms mediate ADCC: perforin or cytolysin produced by the NK cell bind to the infected cell's surface membrane resulting in transmembrane channel formation and osmotic death, and apoptosis. With HIV disease progression there is progressive dysfunction of NK cells which, combined with immune escape variants (conformational) that emerge from effective ADCC responses, are likely to result in a decline in ADCC responses in chronic HIV infection. Of note ADCC activity is higher in LTNP, and the potential to induce or restore this activity is a major focus of current vaccine development.

### 1.1.6 The clinical course of HIV-1 infection and treatment response

HIV-1 infection is characterised in the majority of cases by a prolonged clinically latent state. With disease progression profound immunodeficiency ensues with resultant opportunistic infections (OI) and malignancies and the development of AIDS. The infection is, without treatment, ultimately fatal. This immunodeficiency is, at least in part, reversible with cART and its introduction has led to a dramatic decrease in HIV-1 related morbidity and mortality in the developed world.

In routine clinical care assessment of an individual's immune function tends to be a combination of clinical observation and measuring surrogate immune markers (SIM) such as CD4 T-cell counts. Quantification of CD4 bearing lymphocytes was the first reliable predictor of progression of HIV-1 infection to AIDS or death, with CD4+ T-cell counts <200 cells/μl blood being one of the definitions for AIDS. The significance of the CD4 T-cell count in HIV-1 disease is of great importance, however the full significance of the measured level is still incompletely understood, as undoubtably both qualitative and quantitative factors are at play. More in depth immunological assessment can be performed, particularly measuring T-cell responses (proliferation, cytokine production *etc*.) and T cell phenotype, although there is as yet little evidence of direct clinical correlation, nor evidence that manipulation has resultant clinical benefit. The effects of cART, via virological suppression, on the immune system can be observed through phenotypic analyses of cell surface markers. Significant changes have been seen four weeks post treatment (Autran *et al.,* 1997, Bisset *et al.,* 1998), with increases in memory (CD4+CD45RO+) T-cells and decreases in activated lymphocytes (CD4+HLA-DR+). Such measurements may assist the evaluation of the immune reconstitution that ensues post cART, although functionality is also important, some of which can be inferred by phenotypic changes but not completely. Measurement of activated CD8 T-cells (*e.g.* CD8+CD38+) is a predictor of the development of AIDS and of disease outcome (Liu *et al*., 1997, Douek *et al*., 2009).

The clinical course is an indicator of immune decline as different OI and malignancies tend to occur at 'typical' CD4 T-cell counts. Another clinical correlate of immune function is to measure delayed type hypersensitivity (DTH) as an indicator of overall immune function, but it is now infrequently

utilised in clinical practice. It monitors mainly T-helper responses and typically decreases with increasing progression of disease. DTH responses have been demonstrated to recover with both cART and IL-2 therapy.

### 1.2. cART in Chronic HIV-1 Infection

#### 1.2.1 Background

Since the introduction of cART there has been a dramatic decrease in the morbidity and mortality associated with HIV-1 infection for those individuals with access to therapy (Palella *et al.*, 1998, Lederman *et al.*, 2000). Whilst it has undoubtedly saved countless lives, its widespread, long term clinical use has resulted in the initial optimism regarding its success being tempered with concerns regarding side effects, toxicity, and the emergence of resistance. Spiralling costs of life time therapy and the management of associated complications in HIV-1 infected individuals have increasing implications for costs, healthcare capacity and individual health with the continuing improvement in survival of an aging cohort. These factors have combined to encourage investigation into alternative and adjunctive therapies and different ways of administering cART to minimise side effects and costs. Such approaches range from simple switching of therapies to overcome side effects and avoid potential toxicities, to structured treatment interruptions (STI), to the use of novel therapies such as cytokines and therapeutic vaccinations. Successful alternatives to life long, continuous, triple therapy may additionally expand the number of people world-wide able to access therapies. Much interest has been focused on the role of cART in primary HIV -1 infection (PHI) with a view to elimination of the virus or, failing that, influencing the viral set point and hence improving an individual's prognosis. In several settings this has been attempted with STIs, although data concerning emerging resistance, the possible damage resulting from

increased immune activation and lack of demonstrable benefit, has led to caution regarding this approach. The role of cART in PHI however is outside the scope of this thesis and the focus of this section will be cART in chronic HIV-1 infection. Within this patient group ongoing research is taking place regarding the optimum time to commence therapy, with a shift back towards support for the mantra 'hit early and hit hard'. This is in recognition of the fact that even fully suppressive cART does not allow complete immune reconstitution and that the prevention of damage may indeed be the correct goal rather than treatment after immune damage has occurred. The long term and as yet ill-defined consequences of chronic generalised hyperactivation of the immune system support this approach. The increased event rate in the SMART study (El-Sadr *et al.,* 2008) in those patients who interrupted treatment lends further credence to starting therapy at diagnosis or with a significantly higher CD4 T-cell count than is currently recommended. Recent evidence also suggests this approach may have significant public health benefit via reduced transmissions through reduction in both individual and community viral load (CVL). The currently recruiting international START study should provide more evidence to guide this decision at an individual level. Current UK guidelines for commencing therapy using SIM as a guide recommend a CD4 T-cell cell cut off of 350 cells/μL (Gazzard *et al.,* 2008).

### 1.2.2 Classes of antiretroviral drugs

There are currently 7 classes of antiretroviral (ARV) drugs and 22 licensed drugs available in the UK for the treatment of HIV-1 infection (Table 1.1). These drugs act at distinct points in the HIV-1 life cycle (Figure 1.1)

**Table 1.1 Antiretroviral therapy licensed in the United Kingdom**

| CLASS | DRUG | Abbreviation |
|---|---|---|
| Nucleoside analogues | Zidovudine<br>Didanosine<br>Abacavir<br>Stavudine<br>Lamivudine<br>Emcitricibane | AZT<br>ddI<br>ABC<br>d4T<br>3TC<br>FTC |
| Nucleotide analogues | Tenofovir | TFV |
| Non-nucleoside reverse transcriptase inhibitors | Nevirapine<br>Efavirenz<br>Etravirine | NVP<br>EFV<br>ETV |
| Protease inhibitors | Nelfinavir<br>Saquinavir (soft/hard gel)<br>Ritonavir<br>Indinavir<br>Lopinavir<br>Atazanavir<br>Darunavir<br>Fosamprenavir<br>Tipranavir | NFV<br>SQV SG/HG<br><br>RTV<br>IDV<br>LPV<br>ATV<br>DRV<br>FPV<br>TPV |
| Fusion inhibitors | Enfurvitide | T-20 |
| Entry inhibitors | Maraviroc | MVC |
| Integrase inhibtors | Raltegravir | RGV |

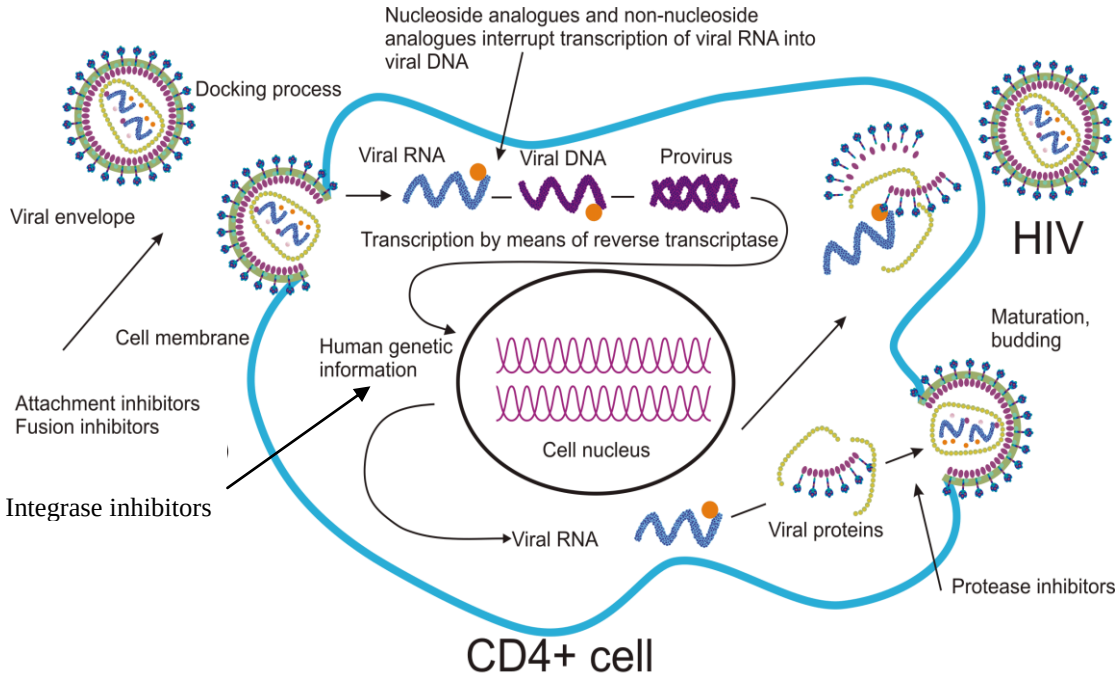



**Figure 1.3 Sites of action in HIV-1 life cycle of the different classes of antiretroviral drugs (N Imami)**

### 1.2.3 Effects of cART initiation

*1.2.3.1 Immune reconstitution*

The use of cART in patients with immunosuppression is associated with significant increases in the CD4 T-cell count in the majority of patients (Staszewski S *et al.,* 1999, Gallant JE *et al*., 2006, Mills AM *et al.,* 2009, Molina JM *et al*., 2010). Introduced in 1996, initially it was unclear whether this quantitative immune restoration would be accompanied by qualitative immune restoration. Clinical evidence of benefit soon became apparent with cases of resolution of Kaposi's Sarcoma (KS) and successful discontinuation of prophylactic therapy for opportunistic infections (OI) (Gill *et al*., 1998, El-Sadr *et al.*, 2000). The CD4 T-cell rise following virological suppression subsequent to cART initiation is due to several mechanisms. Initially there is redistribution and peripheral proliferation (Wei *et al*., 1995, Autran *et al*., 1997, Pakker *et al*., 1998). This redistribution of memory cells from lymphoid tissue follows the cART associated reduction in immune system activation, thus enabling the release of these lymphoid sequestered cells. Peripheral proliferation of existing memory CD4 T-cells also occurs following cART initiation (Wei *et al*., 1995). In both these circumstances these cells have undergone multiple cell divisions and therefore this initial phase is associated with a decrease in TREC levels due to the dilutional effect of cell division (Wei *et al*., 1995, Pakker *et al*., 1998, Mohri *et al*., 2001, Pido-Lopez *et al*., 2003). This phase is followed by thymic production of naïve CD4 T-cells (Autran et al., 1997, Bisset *et al*., 1998, Douek *et al.,* 1998, Pakker *et a*l., 1998, Smith *et al*., 2000). Thymic damage is observed in chronic HIV infection with hypoplasia and decreased function (Stanley *et al*., 1993), and a degree of recovery, in terms of size and function, is observed with cART (McCune *et al*., 1998). Douek and colleagues (Douek *et al*., 1998) demonstrated increased CD4 T-

cells of thymic origin following the initiation of cART as determined by the presence of TRECS. Autran demonstrated an initial rise in memory CD4 T-cells four weeks after starting cART, followed by a rise in naïve cells of thymic origin (Autran *et al*., 1997). Therefore the CD4 T-cell count increases due to both thymic dependent *de novo* production and the thymic independent peripheral mechanisms of prolferation and redistribution.

Improvements in other immunological parameters are also seen, with recovery of some T-cell function. CD4 T-cell proliferative responses to recall antigens can still be detected for a significant period of time in HIV-1 infection (Ballett *et al*., 1998), however they start to decline as the CD4 T-cell count falls (Giorgi *et al*., 1987). Initiation of cART results in some restoration of immune function with, for example, recovery of responsiveness to recall antigens and mitogens (Kelleher *et a*l.1996, Autran *et al*., 1997, Pakker *et al*., 1998), however even this recovery is not absolute as some recall responses are not recovered even after 12 months of therapy (Autran *et al*., 1997). Recovery may be more likely in long term, persistent infections such as HSV and CMV, and less so with short term, intermittent infections and vaccinations such as tetanus (Hardy *et al*., 2003). Immune restoration inflammatory syndrome (IRIS) is the clinical correlate of this recovery, in which immune recovery results in an individual mounting a response to an already present but previously silent infection due to their profound immunosuppression. This is particularly demonstrated in those infections in which the appearance of disease is primarily due to damage resulting from the immune response to the infection, for example Mycobacterium tuberculosis (MTB) and Mycobacterium avium complex (MAC) (Price *et al.*, 2009).

However HIV-1 specific proliferative responses disappear early in infection (Rosenberg *et al*., 1997), and whilst they can be maintained by introducing cART in PHI (Rosenberg *et* al., 1997, Al-Harthi *et al*., 2000) they do not generally re-appear following the commencement of cART in chronic HIV-1 infection (Autran *et al*., 1997, Pontesilli *et al*., 1998). This is likely to be due to several factors; effective cART, by keeping the viral load below the level of detection, reduces the antigenic stimulation provided by the residual virus to such a level that it is ineffective in eliciting an immune response. This is coupled with the lack of complete qualitative immune recovery and persistence of T-cell anergy and subsequent dysfunction; the HIV-1 specific CD4 T-cells may in fact still be present but in an anergic, non-proliferative state (Pitcher *et al*., 1999, Wilson *et al*., 2000). Others suggest inappropriate co-stimulation in a suppressive cytokine milieu may be responsible for the lack of proliferation (Meyaard *et al*., 1993). However some studies have observed a very modest degree of recovery of these specific responses in chronic infection during cART (Kelleher *et al.*, 1996, Li *et al.*, 1998, Angel *et al.* 1998, 2001, Deeks *et al.* 2000, Blankson *et al.*, 2001) and we show in Chapter Three some recovery by switching the drug class utilised in the cART regimen, and in Chapter Four following IL-2 therapy. These initial observations set the stage for several studies of structured treatment interruptions (STI), proposing intermittent auto-vaccination via therapy interruptions as a means to stimulate HIV-1 specific responses. To date these have produced mixed and non-sustained results from the immunological recovery point of view. They carry the additional risk of further immune damage, especially in those patients with low nadir CD4 T-cell counts, and the possible emergence of resistant virus. The results of the SMART study (El-Sadr *et al.,* 2008) have made further trials of STI unlikely. Therapeutic vaccination offers a theoretical

alternative as a source of antigenic stimulation without the inherent risks of STI. The challenge will be to do this in such a way as to avoid or minimise generalised immune system activation with all the associated adverse effects.

Not all patients respond optimally to the initiation of cART. There are four possible outcomes in respect to immunological and virological responses to treatment, two concordant and two discordant or disconnected. The definition and time frame of these outcomes varies but frequently used definitions are described here. Treatment success (TS) results in complete suppression of detectable plasma viral replication (currently <50 copies/ml in routine clinical practice, viral load below the level of detection [VLBD]) and an adequate and concordant rise in the CD4 cell count, arbitrarily defined as >50 cell rise over a six month period. In treatment failure (TF) there is ongoing detectable viraemia and a stable or falling CD4 T-cell count. There are also two possible discordant or disconnected outcomes. One is referred to as a discordant immunological response (DIR) in which the CD4 T-cell count fails to rise despite a fully suppressive cART regimen, usually taken as <50 cell rise after 6 months on cART with a VLBLD. The final possible outcome is referred to as a discordant virological response (DVR), in which despite ongoing detectable virus the CD4 T-cell count continues to rise. These phenomena are examined in more detail in Chapter Three.

The commencement of cART results in multiple phenotypic changes within the T-cell sub-populations. CD4 T-cell counts rise. Initially this is attributed to redistribution, followed by peripheral expansion with an increase in predominantly memory/effector cells (CD45RO) for the first few weeks, followed later by an increase in naive cells (CD45RA) (Autran *et al.*, 1997). There is also evidence of cART increasing the thymic output of naive

precursors (Autran *et al.*, 1997, Douek *et al.*, 1998). Along with peripheral expansion and increased thymic production, decreased apoptosis is thought to play a role in the CD4 T-cell count rise (Dieye *et al*., 2000, Ensoli *et al*., 2000). The markers of activation such as CD38 and HLA-DR decline reflecting control of viral induced hyper-activation (Autran *et al.*, 1997). cART also results in increased expression of the co-receptor CD28 which is essential for effective T-cell signalling; its lack is associated with T-cell anergy and unresponsiveness. Overall cART tends to promote a return towards normality in the immune system, largely by reducing viral burden and hence limiting direct cell killing, activation induced cell death (AICD) and anergy.

The production of naive T-cells and the limited nature of qualitative immune recovery in respect to HIV-1-specific immunity provides the rationale for therapeutic vaccination in the context of fully suppressive cART.

There is a vast array of other changes that have now been described within the immune system occurring after the initiation of cART, but are outside the scope and focus of this thesis.

*1.2.3.2 Clinical*

The immunological recovery due to cART is reflected clinically in the improvement of various HIV-1 related conditions and the decrease in the incidence of HIV-1 related clinical events. DTH to common recall antigens reappear or increase, and dermatological conditions such as psoriasis, HPV and HSV infection and seborrheic dermatitis can all show improvement. Regression of Kaposi's sarcoma has been observed and both primary and secondary prophylaxis for OI can safely be stopped. Although still as yet incompletely defined IRIS reflects this cART induced immune recovery, representing the emergence of an effective immune response to an OI

which due to profound immunosuppression had been silent prior to the initiation of therapy.

There has also been a marked decrease in the incidence of OI occurring following the commencement of cART and its accompanying immunological improvement. In some individuals despite a relative poor CD4 T-cell response there still appears to be a protective effect afforded by the therapy itself, in effect a qualitative improvement despite lack of quantitative advantage. This may be due in part to the effect of decreased immune system hyperactivation by achieving a VLBLD. Howewver, it would appear the strongest predictor of future events is the CD4 T-cell recovery, irrespective of the VL, and this is further explored in Chapter Three.

To further examine the effect of cART on disease outcome and provide the background for this thesis, we undertook a review of mortality in HIV-1 infected patients during the pre-, early- and established cART eras, which is described in Chapter Three.

### 1.2.4 Side effects, toxicities, adherence and resistance

The different antiretroviral drugs have different side effects, ease of administration and genetic barriers to resistance. Major side effects and toxicities include lipodystrophy, hypersensitivity, lactic acidosis, hepatitis, hyperlipidaemia, diabetes and renal, neurological and cardiovascular disease. More common side effects which also frequently affect a patient's willingness to continue with therapy include rash, gastrointestinal side effects and sleep and mood disturbances. Adherence to therapy plays a major role in the success or failure of therapy. Although a patient's health beliefs are thought to be a major influence, pill burden, dose frequency and food restrictions may all influence a patient's level of adherence. The emergence

of drug resistant virus is a major cause of treatment failure and not infrequently results from a patient's poor adherence and is a result of ongoing viral replication in the presence of drug pressure. Some ARV however, such as 3TC and some NNRTIs, have a much lower genetic barrier to resistance than others, requiring only one mutation to confer significant resistance.

### 1.2.5 Limitations

Whilst cART has revolutionised the care of individuals living with HIV-1 there continues to be significant limitations associated with its use. Availability is a key factor, and not only in the developing world, with cost driven waiting lists currently in eight US states (NASTAD, 2010). The degree of quantitative and qualitative immune system recovery is at times sub-optimal. cART is not always fully suppressive with intermittent viral load blips and rebound allowing the emergence of resistant viral strains and re-seeding of latent viral reservoirs. The incomplete recovery of GALT and chronic immune activation results not only in ongoing direct immune system damage but is also linked to several long term complications, including cardiac, renal and metabolic disease. Whilst recognising the revolutionary effect cART has had on the HIV-1 epidemic and individual health, these limitations support the investigation of other treatments and strategies in an attempt to address some of these deficiencies.

## 1.3 Interleukin-2 in HIV-1 Infection

### 1.3.1 Introduction

HIV-1 infection is characterised by progressive immune dysregulation and depletion. This is associated with specific immune system defects and their

characterisation has identified possible therapeutic targets for immunotherapy. Abnormalities in the cytokine milieu are a major defect and interleukin-2 (IL-2) related immune dysfunction in particular is a key factor in HIV-1 related immunodeficiency. However early attempts at immune manipulation by cytokine therapy were largely unsuccessful due to the inability to control viral replication, less than optimal route of administration and the limiting dose related side effects. With the advent of cART and effective suppression of viral replication to undetectable levels there has been renewed optimism regarding the ability of immunotherapy to restore the immune system in HIV-1 infected individuals. Disappointingly, recent large clinical trials (Abrams *et al*., 2009) have failed to show translation of this reconstitution to a clinical benefit in the specific patient groups represented in these studies.

The discussion in this thesis will concentrate on the role of IL-2 in the context of HIV-1 infection. It is highly likely however that just as the immune defects in HIV -1 infection are multifactorial so too will be the solution, and combination immunotherapies are likely to be the way forward.

### 1.3.2 Interleukin-2

Interleukin-2, first described in 1976, was one of the first lymphokines to be identified; it is a major autocrine growth factor that plays a central role in the clonal expansion of activated lymphocytes, by interacting with its specific cell surface receptor (Minami *et al*, 1993). It was first used clinically in patients with metastatic renal cell carcinoma, its sole licensed indication in the United Kingdom, and malignant melanoma. It was first studied in phase I studies in HIV-1 infection in 1983. There has been a wide range of studies looking at different doses (low, intermediate, high), routes of administration

(subcutaneous (sc), intravenous (IV)), formulations (pegylated (PEG)) and schedules (intermittent with varying cycle length, continuous). Numerous combinations of these alternative strategies have been investigated in patients on no, suboptimal and fully suppressive antiretroviral therapy. It is the most extensively studied immune-based therapy in HIV-1 disease. However, the wide variety of doses and schedules and the failure to date to demonstrate clinical and survival advantage, indicate the uncertainty regarding the optimal therapeutic use of this agent.

*1.3.2.1 Basic function*

Endogenous IL-2 is a glycoprotein consisting of an 133-amino acid sequence with a molecular weight of 15-18 kiloDaltons, depending on the degree of glycosylation (Eckenberg *et al.*, 2000). It is produced by activated T lymphocytes and NK cells following antigen activation (Gaffen, 1998). It plays an essential role in the immune response, inducing proliferation and differentiation of T lymphocytes, NK cells and B cells and the production of other cytokines, by interacting with its specific surface receptor (Minami *et al.*, 1993). It influences the development and maturation of thymic lymphocytes. It is chemotactic to lymphocytes and facilitates leukocyte trafficking and adhesion to endothelium (Marincola *et al.*, 1994).

IL-2 is produced following antigenic stimulation of T-cells. This IL-2 activates naive T-cells transforming them into effector cells with the ability to secrete IL-2 and function as antigen-specific CTL (Kaplan *et al.*, 1992, Adachi *et al.*, 1996). This increased production of IL-2 by CTL stimulates T cells, B cells and NK cell proliferation (Kaplan *et al.*, 1992, Marincola *et al.*, 1994, Seder *et al.*, 1995, Adachi *et al.*, 1996, Kinter *et al.*, 1996). IL-2 also upregulates the expression of IL-2R on T and B cells in the presence of antigen, and on NK cells with or without antigen. This results in increased production of IFN$\gamma$, GM-

CSF and TNFα+β (Kaplan *et al.*, 1992, Marincola *et al.*, 1994). There is some evidence that this upregulation of IL-2R on T-cells may also occur without antigenic stimulation (Sereti *et al.*, 2000), and the functionality of these cells is currently under investigation. IL-2 triggers B cells by cross-linking immunoglobulins resulting in IL-2R expression. The IL-2 resulting from the antigen activated T-cells binds to the receptor causing a switch from membrane to secretor IgM and therefore initiating a primary humoral response (Kaplan *et al.*, 1992).

*1.3.2.2 IL-2 receptors*

IL-2 receptors exist as both cell membrane bound and soluble forms. It consists of three basic polypetide sub-units. The different sub-units are expressed on different cells and have differing capacity for intracellular signalling. The sub-units are α (CD25), β (CD122) and γ (CD132). Various combinations of these sub-units result in receptors of differing affinity for IL-2.

The α chain, CD25 (IL-2Rα (p55)) is expressed on activated T and B cells and macrophages, and has low affinity for IL-2 (low affinity receptor (LAR)) (Thèze *et al*., 1996). It plays no role in either IL-2 internalization or signalling (Minami *et al.,* 1993), but facilitates IL-2 binding. It binds IL-2 with a Kd of $10^{-8}$ mmol/L with a dissociation time of seconds.

The β chain, CD122 (IL-2Rβ (p75)) is located on NK cells and activated T-cells. Its intracellular domain consists of three regions, serine-rich, acidic and proline-rich, which are responsible for various intracellular interactions and signals.

The γ chain, CD132 (IL-2Rγ) is expressed on NK and T and B cells and is a member of the cytokine receptor superfamily. It is important for receptor-mediated internalization of IL-2.

Heterodimerization of the β and γ chain results in the formation of the intermediate affinity receptor (IAR), IL-2R βγ (p75/64) which has a Kd of $10^{-9}$ mmol/L and dissociation time of 45 minutes.

Once expressed, CD25 associates with IL-2Rβ chain (CD122) and common γ chain (CD132), which are constitutively expressed on the surface of resting T cells, forming a high affinity receptor (HAR, IL-2Rαβγ (p55/75/64)). This receptor has a Kd of $10^{-11}$ M and a dissociation time of 50 minutes (Thèze *et al*., 1996).

T-cells express α, β and γ subunits and can express high affinity receptors when activated. Activated monocytes only express low affinity receptors.

NK cells are the only cells to constitutively express IL-2R. Those which are CD56+brightCD16- (10% of all NK cells) express both IAR and HAR. When the IAR is exposed to IL-2 for 4-6 hours activation of NK cytotxicity occurs. IL-2 stimulation of HAR for 4-5 days activates NK cells to generate lymphokine-activated killer cells (LAK) *ex vivo* (Brenner *et al.*, 1991, Kaplan *et al.*, 1992). NK cells also mediate ADCC in HIV-1 infection. HIV antibodies bind to NK receptors resulting in lysis by NK cells. Complement mediated NK lysis is increased by IL-2.

*1.3.2.3 IL-2 related defects in HIV-1 infection*

Abnormalities in IL-2 production, function and receptor expression are seen in HIV-1 infection. The TH1 to TH2 shift occurring with disease progression alters the predominant cytokine profile and the high aptotic rates result in a high level of cell destruction.

1.3.2.3.1 Decreased IL-2 production

With HIV-1 disease progression there is decreased IL-2 production and decreased T-cell proliferation (Winkelstein *et al*., 1989, Clerici *et al*., 1993, Lederman *et al*., 1995, Kinter *et al.*, 1996, Clerici *et al*., 1996). As previously

described, with disease progression there is a shift in the immune response from a TH1 to a TH2 response. The production of IL-2 therefore declines and is in fact inhibited by the TH2 cytokine milieu (Clerici *et al*., 1993, 1996, Barker *et al*., 1995, Vyarkaman *et al*., 1995). In addition, it may be that the decrease in IL-2 levels is in part caused by the cross-linking of antibodies to *env* proteins of HIV-1 to IL-2 (Bost *et al.*, 1988). Furthermore, levels of soluble IL-2 receptors (sIL-2R) are increased in HIV infection (Winkelstein *et al*., 1989, Scott-Alzara *et al.*, 1991, Poli *et al.*, 1992, Vyarkaman *et al*., 1995). This increase is usually correlated with a decreased CD4 T-cell count and decreased levels of serum IL-2. The decrease seen in free circulating IL-2 may therefore be due to free IL-2 binding to receptors released from lysed T-cells. Several of HIV-1's 9 regulatory and structural genes influence immune function by directly affecting IL-2 production and function; *tat*, *rev* and *nef* inhibit IL-2 production at the transcriptional level. (Westendorp *et al.*, 1994, Vyarkaman *et al*., 1995, Puri *et al.*, 1995).

1.3.2.3.2 Decreased IL-2R expression

CD25 expression is reduced early in HIV-1 infection with shedding of the protein (Honda *et al*., 1987). *Tat*, *rev* and *nef* downregulate some IL-2R (Westendorp *et al.*, 1994, Vyarkaman *et al*., 1995, Puri *et al.*, 1995), but upregulation of CD122 on CD8+ T cells but not on CD4+ cells (Johnson *et al.*, 1997) is seen.

1.3.2.3.3. Apoptosis

Apoptosis is a normal physiological process whereby cells undergo programmed cell death. In the immune system it is responsible for clonal selection and cell mediated cytotoxicity (Meyaard *et al.*, 1991). It is influenced by many factors, including cytokines. When CD4 T-cells are stimulated by antigen they produce IL-2 which results in clonal expansion.

However with repeated exposure they gradually lose the ability to secrete IL-2. This leads to activation-induced cell death (Smith *et al*., 1988, Meyaard *et al.*, 1991, Adachi *et al*., 1996). With T-cell maturation there is increased expression of Fas antigen and gradual loss of expression of Bcl-2 antigen (Adachi et al., 1996). Fas is a marker for increased susceptibility to apoptosis and correlates with disease progression in HIV-1 infection (Miyawaki *et al.*, 1992).

In HIV-1 infection marked apoptosis is seen in advanced disease (Pandolfi *et al*., 1995, Kinter *et al*., 1996, Clerici *et al*., 1996) as are low levels of endogeous IL-2 (Kaplan *et al*., 1992). The addition of IL-2 *in vitro* blocks spontaneous apoptosis, reducing the down regulation of Bcl-2 expression and therefore increasing cellular survival without inducing cellular division (Adachi *et al*., 1996). *In vitro*, withdrawing IL-2 from gp120 primed lymphocytes results in increased levels of apoptosis (Radrizzani *et al*., 1995, Clerici *et al*., 1996). Additionally, it has been shown that the addition of IL-2 can rescue antigen specific T-cells from radiation induced apoptosis (Mor *et al.*, 1996). This suggests that IL-2 increases the number of CD4 T-cells by preventing apoptosis, with the expansion of existing clones rather than new clones, accounting for the increased CD4 T-cell count (Tepler *et al*., 1993, Wood *et al.*, 1993, Kovacs *et al*., 1995, 1996, Kinter *et al*., 1995, Adachi *et al*., 1996, Jacobson *et al*., 1996) This is consistent with clinical studies of IL-2 showing the expansion of pre-existing (mainly memory) CD4 T-cells (Kovacs *et al*., 1995, Jacobson *et al*., 1996, Kovacs *et al*., 1996). The *in vitro* effect of IL-2 on lymphocyte apoptosis was seen to a greater degree in those with a CD4 T-cell count >200 cells/μL (Adachi *et al*., 1996).

*1.3.2.4 Exogenous IL-2 in HIV-1 infection*

Aldesleukin (Proleukin, Chiron, Emeryville, USA) is currently licensed in the United Kingdom for use in patients with metastatic renal cancer. IL-2 therapy was added to the French HIV treatment guidelines for individuals with low CD4 T-cells, however access to IL-2 therapy was halted in 2007. IL-2 is currently being used in the UK in HIV-1 infection almost exclusively within the context of clinical trials. It is on occasion being used as 'off-license' therapy for HIV-1 infection in the UK.

1.3.2.4.1 Basic structure, pharmacokinetics and function

Aldesleukin is genetically engineered, *Escherichia coli*-expressed recombinant human interleukin-2 (rIL-2) (Chiron). Structurally different from endogenous IL-2, aldesleukin is not glycosylated, lacks a N-terminal alanine residue and at amino acid position 125 the cysteine is replaced by serine. However it is essentially pharmacologically the same as the endogenous cytokine (Doyle, 1985).

The pharmacokinetics of IL-2 is two compartmental and it is rapidly distributed into interstitial fluid. Its distribution half life is 6-20 minutes and its elimination half life is 85 minutes. These are independent of dose and are similar for both intravenous and subcutaneous administration. The mean peak serum concentration is dose dependent and steady state is arrived at in two hours. IL-2 is metabolised by the kidneys.

In patients with metastatic renal carcinoma aldesleukin therapy results in an increase in the levels of sIL-2R. This is associated with increased macrophage activity (Lissoni *et al.,* 1991) and may be indicative of increased immune reactivity (Farace *et al.,* 1995). Increased sIL-2R correlates with increased proliferation of peripheral blood CD25+ T-cells (Hanninen *et al.*, 1991).

Exogenous IL-2 administration results in activation and proliferation of cytotoxic T lymphocytes and NK and LAK cells. Some effects appear dose and schedule–dependent. A transient acute eosinopaenia and lymphocytopaenia, seen immediately after administration, is followed by a rebound increase in the number of both; peaking 1-3 weeks after the start of therapy. The number and activity of the circulating NK cells increase dependent on dose and schedule. A transient increase in both IFN$\gamma$ and TNF$\alpha$ is seen 1-4 hours after administration. There is also an increase in the serum levels of other endogenous interleukins 5, 6, 8 and 10. Production of antibodies specific to rIL-2 has been observed frequently in patients with renal carcinoma, with up to 90% of patients developing antibodies (Whittington *et al.*, 1993, Hanninen *et al.*, 1993), but neutralizing activity is rare (5%) (Hanninen *et al.*, 1991); these patients additionally have lower sIL-2R levels. The clinical significance of these antibodies is as yet undetermined.

1.3.2.4.2 Mechanism of action in HIV-1 infection

IL-2 therapy in HIV-1 infection results in a significant rise in CD4 T-cells. Although still a topic of considerable debate three mechanisms appear to be involved; increased thymic production, peripheral polyclonal T-cell proliferation, with expansion of both memory and naive phenotypes, and reduced apoptosis. Several studies have postulated a direct effect of recombinant IL-2 on the thymus (Plum *et al*., 1987, Reya *et al*., 1998), increasing *de novo* production and maintaining a milieu conducive to support thymocyte growth and selection. Carcelain and colleagues demonstrated an IL-2 associated increase in the levels of TRECS indicating an increase in thymic production of naive cells (Carcelain *et al*., 2003). Additionally, increased CD4 T-cell proliferation accounts for some of the CD4 T-cell expansion observed with IL-2 therapy (Caggiari *et al*., 2001, Natarajan

*et al*., 2002, Pido-Lopez *et al*., 2003). Despite an immediate initial mean increase in T-cell death due to an increased apoptotic rate of both CD4 and CD8 T-cells (Sereti *et al.*, 2001), the apoptotic rate subsequently declines (Caggiari *et al.,* 2000), resulting in an increase in CD4 T-cells. Kovacs and colleagues have also demonstrated that IL-2 therapy induces significant prolongation of CD4 T-cell survival (Kovacs *et al*., 2005).

This expansion of CD4 T-cells is associated with selective induction of the $\alpha$ chain of the IL-2 receptor (CD25) on CD4 T-lymphocytes (Sereti *et al*., 2000). CD4+CD25+ T-cells are also known as regulatory T-cells or Tregs, as they play a key immuno-regulatory role. However those resulting from IL-2 therapy may be qualitatively different to those resulting from antigenic-stimulated expansion (Sereti *et al*., 2000, 2002), being less anergic. IL-2 receptors of high ($\alpha\beta\gamma$) and intermediate affinity ($\beta\gamma$) are up-regulated differentially on CD4 and CD8 T-cells by exogenous IL-2 administration.

This quantitative immunological improvement is accompanied by at least a degree of qualitative improvement. IL-2 results in an increase in delayed type hypersensitivity reactions to such antigens as tetanus toxoid and tuberculin (Carr *et al.*, 1998). Exogenous IL-2 has been shown to increase recall antigen specific CD4 T-cell proliferation (Davey *et al.,* 2000, Levy *et al*., 2003). However the timing of cytokine therapy in relation to antigenic stimulation and recovery of these responses may be crucial (Blattman *et al*., 2003, Hardy *et al*., 2004). Re-exposure to HIV-1 antigens in the presence of IL-2 has been shown to induce HIV-1 specific responses, although sustainability has yet to be demonstrated. However, the clinical benefit of such apparent immune reconstitution remains unknown.

In the context of HIV-1 infection, IL-2 has well described effects on NK cells, humoral immune responses and other cytokines such as IFNγ but these are outside the scope of this thesis.

1.3.2.4.3 Side effects

The side effects of exogenous IL-2 therapy at current doses are predictable and in the majority of cases preventable or controllable by either dose reduction or the administration of prophylactic medication. Earlier trials in HIV-1 infected individuals were limited by the serious, potentially life threatening side effects associated with the high doses of intravenous IL-2 being administered. The majority of side effects are related to the dose and route of administration. There are a wide variety of side effects including pulmonary oedema, cardiac, gastro-intestinal, hepatic and renal dysfunction. The majority are transient and resolve with treatment cessation. The most frequently experienced side effects are flu-like symptoms with chills, fever and malaise, occurring in almost all patients, although rarely to the extent requiring therapy cessation. Fever and chills have been reported as occurring to some degree in up to 100%, although only 5% experienced grade 4 symptoms (Buter *et al.*, 1993). Skin reactions with erythema and pruritis are also common. Inflammation and induration at the injection site are also almost universal and resolve spontaneously over weeks to months. Grade 2-3 local reactions have been reported in 33-100% of patients (Lissoni *et al.*, 1992, Buter *et al.*, 1993, Angevin *et al.*, 1995, Tourani *et al.*, 1996). This can be reduced by site rotation and vigorous rubbing post injection to aid dispersal.

Potentially more serious adverse events include capillary leak syndrome resulting in hypotension and pulmonary and peripheral oedema. This is more commonly associated with high dose intravenous administration (60% mild,

grade 1-2) and is infrequently seen with subcutaneous administration at current doses (0-26%) (Buter *et al.*, 1993).

Other side effects include renal dysfunction, haematological abnormalities (thrombocytopaenia, neutropaenia), nausea, vomiting and diarrhoea.

Hypothyroidism is the major endocrine complication with anti-thyroid antibodies detected in 50%. Thyroid dysfunction is usually temporary and required thyroid substitution in 15% in two trials of patients with renal carcinoma (Buter *et al.*, 1993 Angevin *et al.*, 1995). It has been observed in 10% of patients receiving IL-2 for HIV-1 infection.

Neurolgical and psychiatric disturbances with moderate to severe mental state changes are common and sometimes treatment limiting. Mood depression is not infrequently described, often days to weeks after cycle completion. Musculoskeletal disorders are transient and tend to resolve spontaneously.

As with all drug regimens, acceptability of a treatment takes into account the relationship between the therapeutic benefits to the patient (either real or perceived) and the incidence and tolerability of adverse events. This is of particular relevance when IL-2 is administered to HIV-1 infected individuals with high baseline CD4 T-cell counts.

1.3.2.4.4 Clinical trials

The past 15 years have resulted in a wealth of IL-2 clinical trials showing improvement in SIM and exploring various therapeutic strategies to determine the optimum regimen. Large studies recently conducted were designed to assess whether this gained wisdom translates into improved survival benefit for the individual infected with HIV-1. The major studies are summarised in Table 1.2.

Early trials were hampered by inadequately controlled viraemia and dose limiting side effects. They did, however, demonstrate sufficient benefit to encourage further research into optimum dose, treatment schedules, drug formulation and the method of administration. Trials based on continuous, high dose intravenous IL-2 infusions, limited by serious life threatening toxicities have evolved into subcutaneous, low and intermediate IL-2 dose trials, administered in an outpatient setting, with side effects ameliorated with dose manipulation and prophylactic medication. The resulting data demonstrated SIM benefit at all stages of disease, both with and without cART and without adversely affecting plasma viraemia. Subcutaneous IL-2 has been found to be superior to continuous intravenous and pegylated IL-2 in terms of efficacy balanced against serious side effects and the effect on patients' quality of life (QOL).

In 15 randomised controlled trials of IL-2 therapy in HIV-1 infected patients on ARVT several key factors have now emerged regarding the dosing regimen of IL-2. It appears at present the optimal dose of IL-2 is 7.5 MU bd (Davey *et al.*, 1999). Five day treatment courses at 8 week intervals result in adequate efficacy but with reduced toxicity and avoiding tachyphylaxis (Miller *et al.*, 2001). Additionally greater CD4 T-cell expansion is seen in those patients with a higher baseline CD4 cell count (Davey *et al.*, 1997). The pegylated formulation of IL-2 was found to be inferior in terms of efficacy and similar in terms of side effects (Carr *et al.*1998, Levy *et al.* 1999). The majority of these trials are of IL-2 plus ARVT *vs* ARVT alone, however importantly this does not in all cases equate to virologically effective cART, and in many instances involves mono and dual ARVT.

In a meta-analysis, Emery and colleagues showed in three RCT a decrease in the HIV-1 RNA in 157 patients over 28-31 weeks, maintenance of the CD4 T-

cell rise, and the possibility that there may be decreased risk of disease progression or death in the IL-2 treated patients (Emery *et al.*, 2000). One other RCT has shown a slight decrease in viral load (Davey *et al.*, 2000). The Vanguard study, as discussed in Chapter Five, showed no deleterious effect on viral load in patients receiving IL-2 therapy in the absence of cART (Youle *et al.*, 2006).

Demonstration of the clinical benefit of IL-2 therapy remains an important goal, and two large international, phase three, randomised, controlled, clinical end point studies of IL-2 in HIV-1 positive patients have recently been reported; ESPRIT and SILCAAT (Abrams D *et al,,* 2009). The Evaluation of Subcutaneous Proleukin in a Randomised International Trial (ESPRIT) was a six year study of 4111 patients with CD4 T-cell counts $\geq$300 cells/$\mu$L. The Study of IL-2 with Low CD4 Counts on Active Anti-HIV Therapy (SILCAAT) was a 4 year study of 1695 patients with CD4 T-cell count range of 50-299 cells/$\mu$L. All patients were on cART, although importantly an undetectable VL and minimum CD4 T-cell nadir were not entry criteria. IL-2 cycles were administered for 5 days every 8 weeks; in EPSRIT the dose was 7.5 MIU bd for three cycles and in SILCAAT 4.5 MIU bd for 6 cycles. Additional maintenance cycles were recommended. The main outcome of the study showed that despite a significant and sustained CD4 T-cell increase, no clinical benefit was seen as measured by opportunistic disease or death. In fact, those patients receiving IL-2 did worse in terms of adverse events. This was an unexpected result considering the weight given to the absolute CD4 T-cell count as a reliable SIM, predictive of prognosis. There are many possible explanations for these results. It may be the resultant expanded CD4 T-cell population are in some way defective or poorly functioning, and some evidence exist for this with reported differences observed in the CD4+CD25+

T-cells resulting from IL-2 therapy as compared to 'natural' antigenic stimulation (Sereti *et al*., 2000). Patient selection may also have influenced the outcome as, with no minimum nadir, the potential effector memory T-cell repertoire available for expansion by IL-2 would have been reduced, limiting the potential to protect against OI. Incomplete viral suppression, seen in 20% at enrolment, may also have contributed a dampening effect of any potential IL-2 benefit due to the increased levels of immune activation. In contrast to these explanations it may actually be that we are measuring the wrong SIM, and using that to inadequately drive maintenance therapy; to optimise IL-2 therapy we may need to look elsewhere for alternative or additional SIM, for *e.g.* activation markers, Tregs. Alternatively, it may be that the absolute CD4 T-cell count is merely a surrogate marker for some other immune function which IL-2 does not correct. Finally, it may be that IL-2 does not produce sufficient qualitative immune reconstitution to impact on what is the key outcome of any therapy; disease progression and patient survival. It would, however, be premature to abandon this therapy as the situation currently has several parallels to the early days of mono- and dual-antiretroviral therapy. We still have an incomplete understanding of the pathophysiological mechanisms involved and, as in the past, combination immunotherapy may yield superior benefits as it did with combination ARVT.

**Table 1.2: Summary of IL-2 clinical trials**

| Author, year RCT status | n= | IL-2 dose and route | IL-2 regimen Days/Week/Cycle number | ARVT | CD4 T-cell count (cells/μL) | Viral load (copies/ml) | Main results |
|---|---|---|---|---|---|---|---|
| Kovacs, 1995 | 23 | 8-18 MIU IVI od with dose escalation | 5D/8W 7-25 monthly intervals | yes, not cART | >200 | | 60% of patients with CD4>200 had a CD4 T-cell count rise ≥ 50%, cells were polyclonal with similar Vβ analysis as pre-IL-2 distribution (expansion of the existing repertoire and prolonged survival by decreased apoptosis). Decreased CD8 T-cell activation (HLA-DR, CD38). Progressive increase in CD25+ cells; associated with a higher CD4 cell count. Stable VL overall, peri-cycle increases seen. |
| | 15 | | | | <200 | | Increased VL. No change in CD4. |
| Kovacs, 1996 | 60 | 18 MIU IVI daily | 5D/8W/6C then CD4 driven | 1 or 2 NA | >200 | | Doubling of CD4 T-cell count, sustained > 2 years with repeat cycles. Increase in CD4 %. Stabilisation of CD8 expression of HLA-DR and CD38. No sustained elevation of viral load. |
| Jacobson, 1996 | 16 | 62.5-250k MIU/$m^2$ daily | daily for 6M | ≥1M not cART | 200-500 | | Increase in CD4, no change in VL. DTH to common recall antigens reappeared. |
| Davey, 1997 | 18 | 1.5 or 7.5 MIU bd sc dose escalation | 5D/4 or 8 W/3C | | >200 | | 44% had >200 increase in CD4 T-cell count, further 33% had up to 200 increase. Greatest effect in those with higher baseline CD4. Transient (1 month) rises in VL |
| Carr, 1998 | 115 | A -12 MIU CIVI B - 0.5 MIU sc (PEG) | A - 5D/8W B- day 1 and 3/8W | ≥2 M | 200-500 | | Group A - increased CD4 T-cell count (greater increase at lower VL) and DTH, decreased CD8+HLA-DR, VL unchanged |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Hengge,1998 RCT | 64 | 4.5 MIU bd | 5D/6W or CD4 driven | cART | | | Increased CD4, decreased VL, activation and CD25, increase in DTH, less clinical skin related diagnoses |
| Levy, 1999 | 94 | A-12 MIU IVI<br>B-6 MIU/$m^2$ sc<br>C-2 MIU/$m^2$ PEG IVI | 5D/8W/7C | naïve, started dual ARVT | 250-500 | | Increases in naïve and memory CD4 T-cell count (IV and sc had greater effect *cf* PEG), CD28+ and LPR (mitogen and recall).VL stable |
| Arno, 1999 RCT | 25 | 3 MIU sc | 5D/4W/6C | stable, effective 6M + | ≤250 | ≤500 | Increase in CD4 T-cell count, initially memory then naïve |
| Davey, 1999 | 49 | 1.5 or 7.5 MIU bd sc | 5D/4 or 8W | ≥6W 1, 2 or 3 drugs | ≥500 | | Increased CD4 T-cell and %, dose dependent |
| Lalezari, 2000 RCT | 115 | 1.2 MIU/$m^2$/day sc | daily | cART | <300 | stable | Increase in CD4 % but not absolute count, with preferential expansion of naïve cells. Increase NK cells. Stable VL. Trend to less treatment changes |
| Emery, 2000 | 218 | Metanalysis 3 RCT | | cART | >350 | | Increase in CD4; dose related, decreased VL. Trend to improved clinical outcomes |
| Losso, 2000 RCT | 73 | 1.5/4.5/7.5 bd sc | 5D/8W/6C | cART | ≥350 | | Increase CD4 for 4.5/7.5. Stable VL |
| Davey, 2000 RCT | 82 | 7.5 MIU bd sc | 5D/8W | | 250-500 | <10,000 | Increase in CD4 T-cell count and %, decrease in VL |
| Ruxrungtham, 2000 RCT | 72 | 3 doses | 24W | ARVYT | ≥350 | | Increase in CD4. Stable VL |
| Tambussi, 2001 RCT | 61 | CIV or 7.5 or 3 MIU bd sc | 5D/8W | ARVT | | no requirement | Increase CD4 stable VL and proviral DNA |
| Miller, 2001 RCT | 22 | 4.5MIU bd | 5D/8W/4C or CD4 driven | | >200 | | Increased CD4, no difference b/w groups. Decrease VL |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Marchetti, 2002 RCT | 22 | low dose | | 12M cART | <200 | | Increase CD4 count,increase naïve cells, decrease clinical events, stable VL |
| Katlama, 2002 RCT | 72 | 4.5MIU bd | 5D/6W/4C | cART | 25-200 | <1,000 | Increase CD4, more achieving CD4>200, stable VL |
| Abrams, 2002 RCT | 511 | 4.5 or 7.5 MIU bd sc | 5D/8W then CD4 driven | cART | >300 | | Increase in CD4, no effect on VL |
| deBoer, 2003 RCT | 81 | 12MIU CIV | 3,4 or 5D/ 8W/6C | cART | 100-300 | | Increase in CD4, varied with IL-2 duration, no effect on VL |
| Levy, 2003 RCT | 118 | 5MIU bd | 5D/4W/3C then 8W/7C | cART | 200-550 | | Increase CD4 (naïve,memory), CD25, CD28, NK. Increase response to recall antigen and tetanus immunisation |
| Vogler, 2004 RCT | 115 | 1MIU od sc | 24W | 1,2 or 3 drugs | 300-700 | | Did not prevent CD4 decline |
| Marchetti, 2004 RCT | 15 | | 3C | cART | | | Increase in CD4 count, prolfieration and TRECS cf CD8 |
| Youle, 2006 RCT | 36 | 4.5 or 7.5 MIU bd sc | 5D/8W/3C | none | ≥350 | | Dose dependent increase in CD4 T-cell count. Stable VL |
| Mitsuyasu, 2007 | 204 | CIV or sc | 5D/8W | commenced cART | 50-350 | VL<5000 | Increase CD4. Stable VL. Fewer AIDS diagnoses |
| Durier, 2007 RCT | 131 | bd sc | 5D/CD4 driven | pre and post cART era | | | Increase in CD4, decrease in VL |
| Arudino, 2004 metanalysis | 218 | 1.5/4.5/7.5MIU bd sc | 5D/8W/3C then CD4 driven | cART | ≥350 | | Increase in CD4, dose related |
| Molina, 2009 RCT | 130 | | | no ARVT | 300-500 | | Fewer reached negative endpoints, delaying initiation of cART |
| ESPRIT, 2009 RCT, Abrams | 4111 | 7.5 MIU sc bd | 5D/8W/3C | cART | >300 | | Increase in CD4 T-cell count. No clinical benefit. Increase in grade 4 clinical events |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| SILCAAT, 2009 RCT, Abrams | 1695 | 4.5 MIU sc bd | 5D/8W/6C | cART | 50-200 | ≤10,000 | Increase in CD4 T-cell count. No clinical benefit |

| | | | |
|---|---|---|---|
| MIU | million international units | DTH | delayed type hypersensitivity |
| IVI | intravenous infusion | od/bd | once/twice daily |
| Days/Week/Number | cycle length/cycle interval/number of cycles | sc | subcutaneous |
| D/W/C | days/weeks/cycle number | LPR | lymphoproliferative responses |
| PEG | pegylated | ARVT | antiretroviral therapy |

*Can IL-2 flush viral reservoirs?*

HIV-1 persists as latent, replication competent, integrated virus, despite long term effective cART (Finzi *et al.*, 1997, Wong *et al*., 1997). These infected resting cells produce infectious virions when the cell is activated. The premise therefore is that IL-2 will activate the resting cell, producing infectious virions, but in the presence of cART this will not result in other cells becoming infected. One study which looked at resting, latently infected CD4 T-cells, found a lower frequency of infectious virus in a group of IL-2 treated patients on cART compared in a non-randomised fashion to patients on cART alone (Chun *et al*., 1999). However, virological relapse occurred in all patients who have subsequently stopped cART in this and other studies, indicating eradication had not occurred (Davey *et al*., 1999, Fraser *et al*., 2000, Lafeuiiade *et al*., 2001). In addition as only one in a million PBMCs are latently infected it would require a significant level of activation of all cells to purge the whole reservoir; this degree of immune activation is likely to be detrimental to the host, and toxicity would be a major limiting factor (Fraser *et al*., 2000).

IL-2 however may be useful in promoting control if eradication is not possible. There are three basic observations that underpin this possibility. The central defect in HIV-1 infection is a lack of virus specific T-helper cell response and the main cytokine produced by these cells is IL-2. In chronic infection there are likely to be virus-specific T-helper cells that are not functioning properly (*i.e*. anergic) because of IL-2 deficiency. cART results in the production of new naive cells which due to cART induced lack of antigen (*i.e*. VLBLD) may not change to HIV-1-specific effectors. Therefore IL-2 by stimulating viral antigen from viral reservoirs, may provide this required antigenic stimulus in a

clinically safe way, and may permit reversal of the anergy of the T-helper cells.

In summary IL-2 therapy induces many restorative immunological effects, both quantitative and qualitative. Translation to clinical benefit has yet to be observed. It is likely additional therapeutic immune manipulation and possibly alternative SIM drivers of therapies will need to be identified to realise the promise of IL-2 therapy.

### 1.4 Therapeutic Immunisation in HIV-1 Infection

#### 1.4.1 Background

Expert opinion suggests that an effective preventative vaccine against HIV-1 infection will require both cellular and humoral immunity to achieve protection (Mooij *et al.*, 2001). However, which T-cell responses are critical for protection remain unanswered, furthermore the ability of a vaccine to generate neutralising antibodies remains elusive (MacMichael A, 2006). The added challenge will be to devise a vaccine with sufficient breadth to deal with the different HIV-1 clades encountered around the world. Vaccines can additionally be used therapeutically in those already infected to induce or augment an individual's response to infection, as first proposed by Salk in 1987 (Salk *et al.*, 1987).

Detailed immunological profiling of LTNP has identified various parameters conducive to delayed progression of HIV-1 infection (Deeks *et al.,* 2007). This provides targets for immune manipulation in an attempt to recreate this milieu in HIV-1 infected individuals. The advent of cART has created an environment more permissive to achieving these aims. Prior to the introduction of cART and effective plasma viral suppression, vaccination-induced activation of T-cells provided targets for the virus present in the

plasma. Effective cART has, by viral suppression, minimised this threat, and enhanced its potential as a therapeutic agent.

Initial therapeutic vaccine studies focused on attempts to induce blocking antibodies. Although successful in animal models, trials of agents such as recombinant gp 120 immunogen have proved less successful in humans infected with HIV-1, with no demonstrable clinical benefit (Salk *et al.*, 1987, Ross *et al.,* 2010).

### 1.4.2 Remune: HIV-1 Immunogen

Remune is one therapeutic vaccine candidate which initially appeared promising, although subsequent clinical data has somewhat dampened the early enthusiasm. As is often the case, favourable immune profiles induced by the immunogen have so far failed to translate into appreciable clinical benefit. Remune (Immune Response Corp. (IRC), San Diego CA) is a whole, inactivated, gp120 depleted immunogen suspended in incomplete Freund's adjuvant (IFA) (Moss *et al*. 1994, Trauger *et al*., 1994). It is based on clade A *env* and clade G *gag* from the recombined Zairian primary isolate HZ-321 and grown in the HuT-78 cell line (Moss *et al.*, 1998). It has been administered to more than 3000 trial patients, and appears to be safe and well tolerated (Levine *et al*., 1996, Churdboonchart *et al*., 2000, Chantratita *et al*., 2004). It is administered at doses of 50-400µg total protein, by intramuscular injection at twelve week intervals. Typical reported side effects include flu-like symptoms and local injection site reactions. Several studies have demonstrated improvement in various immunological parameters, including a beneficial effect on CD4 T-cell count and viral load (Turner *et al*., 2001, Sukeepaisarncharoen *et al*., 2001, Chantratita *et al*., 2004). Its use is associated with enhancement of HIV-1 specific delayed type hypersensitivity

(DTH) responses and lymphocyte proliferative responses (LPR) (Valentine *et al.*, 1996, Churdboonchart *et al*., 2000, Turner *et al*., 2001, Robbins *et al*., 2003). It increases levels of IFNγ (TH1 cytokine) and β chemokines (Moss *et al*., 1997, Valentine *et al*., 1998). It has been shown to augment cell mediated immune (CMI) responses to autologous virus and increased antibody reactivity has been described; both strength and breadth of Western Blot (WB) reactivity (Churdboonchart *et al*., 2000).

The first clinical study of Remune in 1994 was a phase one, dose ranging study in ARV naïve HIV-1 infected individuals which showed improved HIV-1 specific DTH and antibody response (Turner *et al.*, 1994). A subsequent study (Levine *et a.*, 1996) demonstrated improved DTH in approximately 50% of the 25 patients, who then went on to have a decreased rate of clinical events and death. This association was potentially biased by the possibly more conserved immune function of those individuals in the more reactive group. An IRC study in 15 patients, demonstrated increases in IFNγ, β chemokines and LPR to HIV-1 specific antigens (Moss *et al.*, 1997). A Thai study of 29 ARV naive individuals, 28 infected with clade E, also showed improvement in WB reactivity in those receiving Remune. A subsequent larger, double-blinded, adjuvant-controlled trial demonstrated increases in CD4 T-cell counts and HIV-1-specific immunogenicity (DTH and WB) (Churdboonchart *et al*., 2000). Other Thai studies have shown stabilisation of body weight, viral load and CD4 T-cell percentage (Sukeepaisarncharoen *et al*., 2001, Chantratita *et al*., 2004). Valentine and colleagues reported increased levels of *gag*-specific T-helper-cell responses (Valentine *et al.,* 1996) and β chemokines (Valentine *et al*., 1998)) comparable to that seen in LTNP, along with a greater proportion of individuals achieving viral loads BLD in those receiving Remune. Others

have also observed increases in HIV-1 specific CD4 T-cell proliferative responses (Moss *et al.*, 2000, Robbins *et al.,* 2003).

The largest study to date is a phase three, multicentre, double-blind, placebo controlled, randomised, clinical endpoint trial in 2527 ARVT naïve HIV-1 infected individuals (Kahn *et al.*, 2000). HIV-1 progression-free survival was the primary endpoint with secondary endpoints being overall survival, HIV RNA, CD4 T-cell count and percentage, body weight and immunogenicity. The only difference observed was an increase in the mean CD4 T-cell count, and no difference in clinical outcomes was observed. The publication of these results was controversial due to author disagreement with the sponsoring company regarding analysis and interpretation of the results. Results from a prospectively identified sub-group (n=252) by Moss and colleagues (IRC) revealed the vaccinated group had a significantly greater decline in plasma viral RNA, both in the speed of decline and the percentage of individuals becoming undetectable, and enhanced lymphoproliferative response (LPR) to HIV-1 specific antigens (Moss *et al*., 2000).

Remune does appear to have potentially beneficial effects. Whether this translates to clinical advantage remains to be seen. Enhancement of the effects observed to date may also be of benefit. We therefore included Remune in the study described in Chapter Four of this thesis to observe its effect on T-cell phenotype and whether these were further influenced by IL-2 immunotherapy.

## CHAPTER TWO: MATERIALS AND METHODS

### 2.1 Study populations and ethical approval

Participants were HIV-1 infected patients attending the St. Stephen's Centre, Chelsea and Westminster Hospital. Different study populations were used for each study. Details specific to each study relating to participant characteristics are contained in the relevant chapter. Where required ethical approval was obtained for each study and all patients gave written informed consent.

### 2.2 Blood samples

All procedures were carried out according to health and safety regulations. Blood samples were collected at different time points for the different studies and are detailed in the relevant chapter. Peripheral venous blood was collected into sodium heparin and ethylenediamine tetraacetic acid (EDTA) containing vacutainer tubes (Becton Dickinson, Oxford, UK). Heparinised blood was used for lymphoproliferative, $Cr^{51}$ release and ELISPOT assays. EDTA preserved blood was used for flow cytometric and viral RNA and proviral DNA analysis.

### 2.3 Containment Level III (Cat III) training

All experiments involving HIV-1+ blood were performed in the Containment Level 3 Suite in accordance with the code of practice and the guidelines recommended by the Advisory Committee on Dangerous Pathogens (ACDP). Briefly, the Cat III training involved 30 hours of observation followed by 30 hours of supervised work, after which authorization to work in the Cat III suite was granted upon successful completion of an assessment.

### 2.4 Plasma separation

Plasma was separated by gravity or centrifugation for 10 minutes at 1800 rpm, aliquoted into 1/5ml skirted tubes (Elkay, Costello, Ireland) and stored at –80°C. The cell fraction was saved for the separation of peripheral blood mononuclear cells (PBMC).

### 2.5 Peripheral blood mononuclear cell (PBMC) separation from whole blood

The plasma volume removed was replaced with equal volume of RPMI 1640 medium (Sigma, Poole, UK) supplemented with 100IU/ml penicillin, 100μg/ml streptomycin and 2mM L-glutamine (all Sigma). The cells were layered over 20mls of Histopaque (Sigma) and centrifuged at 2200 rpm for 20 minutes without brake. The interface, PBMC, was collected, transferred into sterile falcon tubes and diluted with sterile phosphate buffered saline (PBS) (Sigma), after which cells were washed at 1800 rpm for 10 minutes. The cells were counted (see 2.5.2) and aliquoted as required for functional and phenotypic assays. Remaining PBMC were frozen and stored in liquid nitrogen (see 2.6).

#### 2.5.1 Cell counts and viability

The KOVA method was used for enumeration and determination of PBMC viability. This involved the transfer of a 50μl aliquot of cells into an eppendorf tube and the addition of 50μl of 0.4% Trypan blue solution (Sigma). After cells were mixed by pipetting, approximately 10μl was placed on a KOVA Glastic® slide (Hycor Biomedical Inc, Edinburgh, UK). The slide was placed on an inverted microscope and the PBMCs were counted using the x40 lens. The number (n) of cells in 9 small diagonal squares were counted and multiplied by 2 (Trypan blue dilution factor 1:1). This number was multiplied by $10^4$ (chamber factor) to get the number of cells per ml in the cell suspension. A

final multiplication by 50 (volume) gave the total cell yield in the 50ml falcon tube. Thus, total cell count = n x 2 x $10^4$ x 50. Cells were then centrifuged at 1500rpm for 10minutes with brakes set to 9. Once the supernatant was discarded the cell pellet was resuspended at $10^6$ cells per ml in sterile PBS. This was then used for functional assays (LPR as detailed below) and phenotypic analysis (flow cytometry, as detailed below).

**2.6 Cryopreservation and thawing of PBMC**

PBMC were resuspended using 2 mls of cold ($4^0$C) foetal calf serum (Sigma) supplemented with 10% dimethyl sulfoxide (Sigma). One ml was aliquoted into cryotubes (Fisher Scientific, Loughborough, UK) on ice. These were placed in a Naglene 'Mr Frosty' box (Fisher Scientific) and stored at –$80^0$C for 24 hours. After 24 hours the vials were moved on dry ice into liquid nitrogen storage.

Thawing was performed as rapidly as possible. Cells were conveyed on dry ice and thawed and resuspended in 50mls of cold phosphate buffered saline (PBS, Sigma). They were spun immediately at 1800rpm at $4^0$C for 10 minutes. Cells were then washed twice in PBS.

**2.7 Lymphocyte subset quantification**

The Epics XL-MCL (Beckman Coulter) was used for four colour flow cytometric analysis of total $CD3^+$, $CD4^+$, $CD8^+$, $CD16^+/CD56^+$ (NK cells) and $CD19^+$ (B cells) lymphocytes, using whole blood. Murine monoclonal antibodies (mAbs) used were anti-human CD3, CD4, CD8, CD16/CD56 and CD19 (TetraOne, Beckman Coulter, High Wycombe, UK). These tests were conducted by the clinical laboratory staff at Chelsea and Westminster Hospital.

### 2.8 Plasma viral RNA assay

Plasma HIV-1 RNA copy levels were measured using the Quantiplex HIV RNA 3.0 Chiron branched DNA (bDNA) assay with a detection limit of 50 copies/ml (Chiron Diagnostics, Halstead, UK). These tests were conducted by the clinical laboratory staff at Chelsea and Westminster Hospital.

### 2.9 Immunophenotyping: Flow cytometric analysis

PBMC were processed either as fresh samples or as batched cryopreserved samples (see 2.6). Optimal volumes of antibodies were used as assessed independently by Imami, Sullivan and Burton (London, UK) and Autran and colleagues (Paris, France) as part of the INITIO study (Yenni *et al.,* 2006). 10 μl of FITC and PE conjugated mAbs and 5μl for PE-Cy5 conjugated mAbs (Table 2.1) were incubated with 100 μl of EDTA sample ($1\times10^6$ cells) for 30 minutes on ice in a darkened CAT III hood. PBMC were washed with PBS at 1500 rpm for 5 minutes and resuspended in 500 μl of 2% paraformaldehyde in PBS. Samples were analysed within 24 hours of preparation (refrigerated and covered if not acquired immediately). All reagents were kept in the dark at $4^0$C.

Three colour flow cytometry acquisition and analysis was performed on FACSCalibur with Cell Quest software (both Becton Dikinson). A combination of forward and side scatter density plots and CD3+ plots were used for gating. Two further gates were set around the CD4+ and CD8+ T-cell populations. 10,000 gated events were collected. Analysis was performed using logical gating in Cell Quest software. Conjugated isotype matched controls were used to set compensation and voltage.

**Table 2.1 Murine monoclonal antibodies used for three colour flow cytometric analyses** (Beckman Coulter, Marseilles, France).

| SPECIFICITY | MOUSE ISOTYPE | FLUORO-CHROME | CLONE | REFERENCE |
|---|---|---|---|---|
| CD3 | IgG1 | FITC | UCHT1 | IM 1281 |
| CD4 | IgG1 | PC5 | 13B8.2 | IM 2636 |
| CD8 | IgG1 | PC5/FITC | B9.11 | IM 2638/IM 0451 |
| CD45RA | IgG1 | PE | ALB11 | IM 1834 |
| CD45RO | IgG2a | PE | UCHL1 | IM 1307 |
| CD62L | IgG1 | FITC | Dreg 56 | IM 1231 |
| HLA-DR | IgG1κ | PE | Immu-357 | IM 1638 |
| CD38 | IgG1 | PE | T16 | IM 1832 |
| CD28 | IgG1 | PE | CD28.2 | IM 2071 |
| CD25 | IgG2a | PE | B1.49.9 | IM 0479 |
| CD122 | IgG1 | PE | CF1 | IM 1978 |
| MsIgG1 | IgG1 | FITC | 679.1Mc7 | IM 0639 |
| MsIgG1 | IgG1 | PE | 679.1Mc7 | IM 0670 |
| MsIgG1 | IgG1 | PC5 | 679.1Mc7 | IM 2663 |
| MsIgG2a | IgG2a | PE | U7.27 | IM 0671 |

FITC - fluorescein isothocyanate, PE - phycoerythrin, PC5 - PE-cyanine 5.

### 2.10 Proliferation assays

Proliferation assays were set up using a standardised protocol. Recall antigens, mitogens and cytokines were suspended in 10% AB plasma/RPMI (Sigma), at the concentrations below. 100µL was added to 96 well round bottomed microtiter plates (Greiner Laboritechnik, Stonehowe, UK). Each antigen was tested in triplicate, and two triplicates of TCM were set up as negative controls. Freshly separated PBMC were added to each well giving a final concentration of $10^5$cells/well. Antigens and mitogens used at concentrations as listed (Imami *et al*., 1999):

Baculovirus-derived recombinant p24, EVA no.620, 10µg/ml

Baculovirus-derived recombinant gp120, EVA no 646, 10µg/ml

*E.coli*- derived recombinant nef, EVA no 650, 10µg/ml

Influenza A, Central Public Health Lab (CPHL), 1/40

Herpes simplex virus, CPHL, 1/40

Varicela Zoster, CPHL, 1/40

Cytomegalovirus, Behring, 1/40

Toxoplasma antigen, Behring, 10µg/ml

Purified protein derivative, Sigma, 10µg/ml

*Candida albican* NIBSC

Concanavlin A, Sigma, 10µg/ml

Pokeweed mitogen, Sigma, 10µg/ml

Recombinant IL-2, Boehringer, 20 and 100 U/ml

Plates were incubated for 5 days at $37^0$ and 5%$CO^2$. On day 5, 100 µl of supernatant was collected and stored at -20°C for subsequent cytokine measurement. Each well was then pulsed with 1 µCi $^3$H-methyl thymidine ($^3$H-TdR; Amersham International, Amersham, UK) and 16 hours later cells were harvested onto glass fibre filtermats (Wallac Oy, Turka, Finland). Proliferation as measured by beta particle emission from $^3$H-TdR incorporation was evaluated by liquid scintillation spectroscopy using a 1205 Betaplate counter (Wallac) (Imami *et al*. 1994). Results are expressed as stimulation index (SI) and as mean counts per minute (cpm) for triplicate cultures, with percentage error of the mean <15%. The SI was calculated by dividing the experimental beta particle cpm by the background cpm. A positive response is defined as a SI of five or more. Control wells, for calculation of background activity, contained PBMC only.

Recombinant HIV-1 antigens were obtained from Medical Research Council Centralised Facility for AIDS Reagents (NIBSC, Potters Bar, UK).

Performed by Dr Nesrina Imami and AKS.

### 2.11 Measurement of IL-2 and IL-4 production

Supernatant (50 µl) from proliferative cultures was transferred to a pair of 96-well round bottomed plates as triplicates for measurement of IL-2 and IL-4 using indicator cell lines CTLL-2 (European Collection of Animal Cell Culture, Salisbury, UK; ECACC) and CT.h4S (a generous gift of W. Paul, Bethesda, MD). 50 µL CTLL-2 at a concentration of $10^3$ cells/well or CT.h4S at a concentration of $5x10^3$ cells/well was added giving a final volume of 100 µL. After 24 h wells were pulsed with $^3$H-TdR and harvested as described above (Imami *et al*., 1994, Fessel *et al*., 2000).

Performed by Dr Nesrina Imami and AKS.

### 2.12 Latent proviral DNA

HIV-1 proviral DNA was measured utilising spectophotometric PCR methodology (Hardy *et al*., 1999) with an analytic sensitivity of 10 copies/µg of total cellular DNA.

Performed by Dr Nesrina Imami, Dr Antonio Pires, Dr Catherine Burton and AKS.

### 2.13 Statistical analysis

The statistical anlaysis used for each study is detailed in the relevant chapter. All statistical analyses were conducted in collaboration with the onsite statistician, Dr Sundhiya Mandhalia.

# CHAPTER THREE: THE IMMUNE SYSTEM RESPONSE TO COMBINATION ANTIRETROVIRAL THERAPY (cART)

## 3.1 Introduction

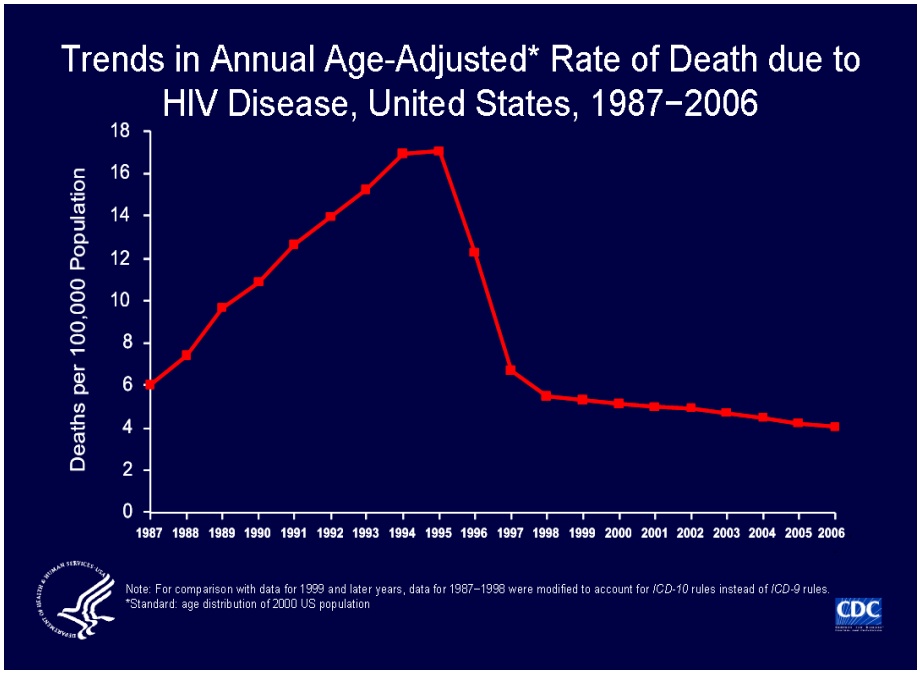
A wide variety of responses are observed when individuals are commenced on cART; between individuals and between and within the different drug classes. Factors influencing these differences include patient related factors such as genetics, adherence and co-morbidities, viral factors such as viral clade and virulence and drug related factors such as tolerability, regimen complexity and effect on the immune system. In this first section of my thesis my aims, related to cART, were threefold; firstly to describe the changing causes of mortality following the introduction and widespread use of cART, secondly to describe and quantify the various responses to cART in terms of virological and SIM in a cohort of ARVT naïve patients and finally to examine whether differences were seen between different classes of drugs both on surrogate markers and HIV-1 specific immune responses. I carried out a two centre audit of all cause mortality in patients during three distinct periods relating to cART evolution, I then analysed virological and SIM outcomes in a large patient cohort initiating cART and, finally I measured HIV-1-specific responses in a small group of patients switching therapy from a protease inhibitor (PI) to a non-nucleoside reverse transcriptase inhibitor (NNRTI) based regimen. The results are presented separately within this chapter.

## 3.2 Effect of cART on all cause mortality – Mortality Audit

### *3.2.1 Introduction*

The introduction of cART in the developed world has resulted in a substantial decrease in the morbidity and mortality associated with HIV-1 infection (Pallela *et al.*, 1998, Lederman *et al.,* 2000) (Figure 3.1).

**Figure 3.1 HV-1 mortality rates in the USA (CDC)**




The causes of death however are changing, with non-AIDS related conditions increasing in importance (Lucas *et al.*, 2008, Mocroft *et al.*, 2010, Adih *et al.*, 2010) (Figure 3.2).

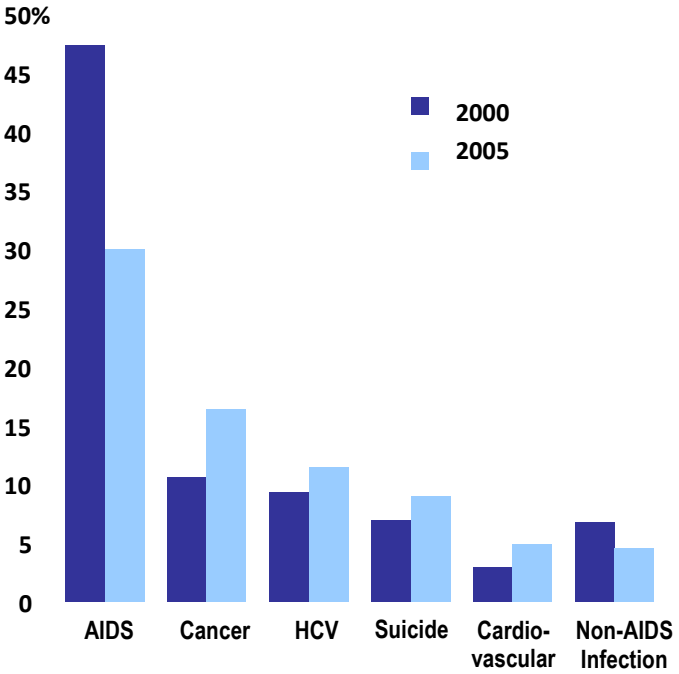



**Figure 3.2 Changing patterns of mortality among HIV patients in France 2000-2005 (Lewden *et al.*, 2007)**

Furthermore, whilst AIDS related malignancies are decreasing, non-AIDS cancers are increasing in prevalence (Simard *et al*., 2010), and cART itself has now been linked to non-AIDS related conditions, such as cardiovascular disease (Friis-Møller *et al*., 2003). Triple combination therapy, initially PI then NNRTI based, became available in the UK towards the end of 1994 and more widely by the end of 1996. Since that time its use has become widespread resulting in longer survival (HPA, 2009). We undertook a retrospective, observational audit to examine characteristics of patients dying with HIV-1 infection at two centres in the UK, their cause of death and ARVT, and to observe the changing causes of mortality following the introduction and wide spread use of cART. This was performed to provide the background to this thesis in terms of clinical outcome data to demonstrate the effect cART has had on mortality but also to highlight potential ongoing qualitative immune defects. The aim was to establish if patients on effective cART were dying from conditions which may be related to a qualitative immune deficiency from incomplete restoration of immune function.

### *3.2.2 Patients and Methods*

We identified three distinct time points; November 1994 when triple combination therapy was first prescribed at Chelsea and Westminster Hospital (CWH), November 1996, two years after the introduction of cART, and September 2002, 6 years after its widespread use when cART was accepted as best clinical practice. At one centre (CWH) we identified 100 consecutive deaths of patients attending the centre immediately prior to the first time point and subsequent to the second two, thus defining three distinct periods; pre-cART era (era A, 11.5.94-30.11.94), initial cART (era B, 4.11.96-19.5.98) and established cART (era C, 8.6.2000-27.9.2002). We subsequently

identified all deaths occurring during the same time periods at the second centre. We performed a data base and case note review. Additional information was sought from alternative sources including treating physician, other hospitals, hospices and Coroner's offices. We also obtained data from both centres to define general clinic demographics.

*3.2.3 Results*

A total of 397 deaths were reviewed from the three eras; 133 era A, 133 era B and 131 era C. The eras progressively increased in length following the introduction and subsequent widespread use of cART, reflecting the increasing time period required for the 100 deaths to occur. Patient characteristics are detailed in Table 3.1.

The proportion of women dying increased over time, such that in era C women were over-represented compared to the general clinic population, and also in those newly diagnosed. Patients in era C were dying with increasingly less advanced disease; with higher CD4 T-cell counts (median 20 *vs* 35 *vs* 116 cells/μL, A *vs* B *vs* C, $p<0.0001$), lower viral loads and fewer patients having been previously diagnosed with AIDS (Table 3.1).

**Table 3.1 Patient characteristics at the time of death for the three eras**

| | A | B | C |
|---|---|---|---|
| Era duration (months) | 6 | 17 | 27 |
| Deaths (n) | 133 | 133 | 131 |
| Sex:<br>Cohort M:F (%)<br>Clinic M:F (%) | <br>97:3<br>94:6 | <br>92:8<br>91:9 | <br>84:16<br>88:12 |
| Age (years) | 39.3 | 41 | 41 |
| Total clinic population (n) | 1871 | 3106 | 5019 |
| Death rate (%) | 7.3 | 5.1 | 2.6 |
| Risk category (%)<br>cohort [clinic population]<br>MSM<br>Heterosexual<br>IVDU | <br><br>90 [84]<br>2 [6]<br>7 [7] | <br><br>78 [80]<br>11 [10]<br>11 [7] | <br><br>61 [72]<br>31 [23]<br>8 [8] |
| Median CD4 T-cell count cells/μL blood | 20<br>(0-252) | 35<br>(0-770) | 116 *<br>(0-1379) |
| Median Viral Load (copies/ml plasma) | NA | 89811 | 1925 |
| VLBLD n (%)<br><400 copies/ml<br><50 copies/ml | <br>NA<br>NA | <br>10 (8)<br>NA | <br>51 (39)<br>37 (28) * |
| Previous AIDS Diagnosis (%) | 93 | 77 | 44 * |
| On cART (%) | 0 | 35 | 55 |
| New HIV Diagnosis n (%) | 3 (2) | 12 (9) | 11 (8) |

* $p < 0.001$

The causes of death changed over this period (Table 3.2), with lymphoma and other cancers becoming more frequent as a cause of death. Liver disease, in particular co-infection with hepatitis B or C, also increased as a cause of death; despite the survival from all these conditions improving in the HIV-1 infected population over this time period. After the introduction of cART, *Pneumocystis cariniii* pneumonia (PCP) (now renamed *Pneumocystis jiroveci*) continued as a significant cause of death for several years particularly in those whose HIV-1 status was unknown at presentation, however this phenomenon appeared to be declining. Of patients who died from PCP 1/14 (7%) in era A, 10/13 (77%) in era B and 4/9 (44%) in era C were in patients who presented unaware of their HIV-1 infection. Kaposi's sarcoma (KS) decreased as a cause of death, reflecting the beneficial effect of cART

on KS. Sepsis, particularly in association with pulmonary infection, increased. Deaths recognised as due to ARVT have increased since the introduction of cART, but not during the cART era, despite the more widespread use.

**Table 3.2 Causes of death in the three eras**

| **Causes of death** | **A** | **B** | **C** | p |
|---|---|---|---|---|
| KS | 13 | 7 | 2 | 0.004 |
| Lymphoma | 2 | 9 | 10 | 0.03 |
| PCL | 6 | 5 | 2 | 0.015 |
| Other Cancer | 0 | 0 | 11 | 0.0001 |
| OI* | 28 | 18 | 15 | 0.06 |
| Sepsis | 10 | 10 | 18 | 0.056 |
| Suicide | 0 | 4 | 8 | 0.018 |
| Liver | 2 | 3 | 11 | 0.0001 |
| ARVT | 0 | 2 | 3 | 0.13 |
| *of which PCP | 14 | 13 | 9 | |

PCL = primary cerebral lymphoma, OI= opportunistic infections, KS= Kaposi's sarcoma PCP= *Pneumocystis carinii* pneumonia

In era B, 44 (33%) patients were receiving cART; for 12 (27%) it was their first regimen and 10 (27%) had a VLBLD, for era C this was 72 (55%), 21 (38%) and 37 (51%) respectively.

In era C, 37 (28%) patients died on voirologically successful cART with a VLBLD (50 copies/ml). Of these patients the causes of death were 6 Non-Hodgkin's Lymphoma (NHL), 6 other cancers, 6 liver disease, 4 sepsis and 2 ARVT; there were no opportunistic infections (OI). In the 21 patients dying with a detectable VL, 14 had been on therapy for less than 3 months. In those 59 patients in era C not on cART, 27 had been on cART previously. In the 32 with no record of previous cART, 11 were new diagnoses.

*3.2.4 Discussion*

cART has substantially reduced the mortality of those individuals infected with HIV-1 (Pallela *et al.*, 1998, Lederman *et al.,* 2000). However it still has a significant mortality associated with it and the causes of death have

changed (Friis-Møller *et al*., 2003, Lucas *et al*., 2008, Mocroft *et al*., 2010, Adih *et al*., 2010, Simard EP *et al*., 2010). This reflects the longer survival of patients, the influence this has on the diseases they are susceptible to, the stage of disease at which they are maintained by cART, and the cART regimen they are taking. Similarly to others (Simard *et al*., 2010), we observed a striking increased proportion of lymphoma and other non-AIDS cancers as a cause of death, which may reflect incomplete qualitative immune recovery. We also observed a proportional increase in co-morbidities, such as hepatitis C related liver disease, as a cause of death. There was direct cART associated mortality (hypersensitivity, hepatic failure) although, despite more widespread use, this did not increase, and this may reflect increasing physician experience in recognising adverse reactions and more effectively managing them. There were no deaths identified as being due to cardiovascular disease. It is important to note that patients with virologically successful cART are still dying from HIV-1 related illnesses. However a large number of patients who die, do so shortly after their HIV-1 diagnosis and the commencement of potentially life saving therapy, representing potentially missed opportunities for immune recovery. This is highlighted in the BHIVA mortality audit showing one third of deaths occur in individuals diagnosed too late to initiate effective cART (Lucas *et al*., 2008). However, the fact that patients are still dying with less advanced disease as measured by their CD4 T-cell count highlights the gap between quantitative and qualitative immune recovery on cART.

## 3.3 Study One: Discordant CD4+ T-lymphocyte responses to the initiation of combination antiretroviral therapy in anti-retroviral naive HIV-1 infected individuals

### *3.3.1 Introduction*

The initiation of cART results in the majority of patients achieving control of HIV-1 infection as evidenced by a plasma viral load below the level of detection (VLBLD) and a degree of immunological recovery as shown by an increase in the CD4+ T-cell count (Staszewski S *et al.,* 1999, Shafer RW *et al*., 2003, Squires K *et al*., 2004, Gallant JE *et al*., 2006, Mills AM *et al.,* 2009, Molina JM *et al*., 2010). However, in a proportion of patients this ideal response to therapy (treatment success, TS) does not occur (Gilson *et al.*, 2010). Three other possible outcomes are described; treatment failure (TF; detectable viral load and lack of significant CD4 T-cell rise), discordant immunological response (DIR; VLBLD and lack of significant CD4 T-cell rise) and discordant virological response (DVR; detectable viral load and a significant rise in CD4 T-cell count). The frequency at which these different discordant responses have been reported varies considerably due to different definitions, periods of follow-up and proportions of the cohorts who have previously been exposed to ARV therapy, albeit not necessarily optimal cART (Picketty *et al*., 1998, Perrin *et al*., 1998, Barreiro *et al*., 1999, Grabar *et al*., 2000).

### *3.3.2 Patients and Methods*

We identified all HIV-1-infected, ARV naive individuals attending a single out-patient department who were commenced on cART; defined as a combination of at least three ARV agents. To be eligible, patients had to have CD4 T-cell count and viral load results available at baseline (BL, including up to 2 months prior to initiation of cART) and at 12 months (+/- 2

months, 12M). Data were also obtained for the 2 years prior to initiation of cART and at 24 months post baseline where available. Responses to therapy for the definition were taken at 12 months. TS was defined as an undetectable viral load (<500 copies/ml) and a CD4 T-cell count rise of >50 cells/μL and TF as detectable plasma viral load and a rise of <50 cells/μL blood. For the discordant outcomes, DIR was defined as VLBLD and a CD4 T-cell rise of < 50 cells/μL and DVR as detectable virus and a CD4 T-cell rise >50 cells/μL. Over the period of the study the definition of VLBLD changed in the clinic to <50 copies/ml and so data are presented for 500 copies/ml for the whole cohort and additionally for 50 copies/ml where available. Data were also collected on demographics and ARVT combinations, including any changes to the combination, although the data is presented as intention to treat analysis. Clinical events, defined as disease progression or death were also recorded.

*3.3.3 Statistics*

Demographic characteristics are summarized using the descriptive statistics by treatment outcome quadrants. Where quantitative data are hyper geometrically distributed, median with inter-quartile ranges are presented while Gaussian normal data are summarized using the mean and standard deviation. Qualitative data are summarized using number of subjects with respective percentages. The primary efficacy endpoints are evaluated using the MIXED procedure in SAS (Cary, USA). The statistical analysis that is used considers two main characteristics of the longitudinal data and these need to taken into consideration: repeated observations for a subject are likely to be correlated, and missing data; whether due to drop out, patient not attending for their scheduled study visit or data missing for other reasons such as laboratory errors. These types of unavailable data in the successive

responses may induce a bias. As a reason an analytic approach taken took these types of considerations into account and the statistical method used was the generalized linear MIXED model.

In addition, as longitudinal data were available for study time points, and since multiple assessments of the same subject were available at different time points, within subject responses are expected to be correlated. This correlation was accounted for in this analytical method used.

Linear mixed models was used to derive time adjusted changes in the surrogate immunological markers such as CD4 T-cell count by treatment outcome quadrants of TS, DIR, DVR and TF. MIXED procedure in SAS was used by fitting values of CD4 T-cell count from all study time points as a dependent variable. Independent variables included the fixed effects of treatment outcome quadrants, study visit time points and treatment outcome quadrants by time interaction. An unstructured covariance matrix was used to model the within patient errors.

Estimates of change in CD4 T-cell count since pre start of cART and since starting cART, used as baseline, were obtained from treatment outcome quadrants by time interaction. Trends over time are presented as point estimates and described with 95% CI.

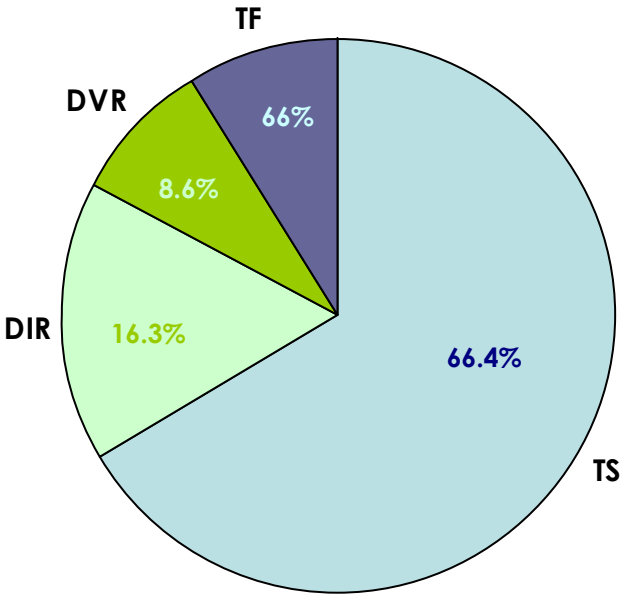
*3.3.4 Results*

One thousand, three hundred and sixty-six patients commenced cART during this period (September 1996 - September 2002), 1141 were eligible for inclusion in the analysis.

Of 1141 patients, 757 (66.4%) achieved a successful treatment response by 12 months and 100 (8.8%) experienced treatment failure. A DIR was seen in

186 (16.3%) and a DVR in 98 (8.6%). Eighty-three percent (943) therefore had a VLBD at 12 months (p<0.001) (Figure 3.3).

**Figure 3.3 Treatment outcome in 1141 HIV-1 infected individuals 12 months after commencing cART**




In those patients (n=904, 79.2%) for whom a more sensitive assay was available, 758 (83.8%) achieved an undetectable viral load (<50 copies/ml) at 12 months. For each group the percentage of those with a more sensitive assay available was similar apart from being slightly reduced in the DVR group; 80.7 *vs* 79.0 *vs* 68.4 *vs* 79.0 for TS *vs* DIR *vs* DVR *vs* TF respectively. The resulting proportions in each outcome group as expected are decreased in TS and DIR and increased in DVR and TF, although this is not significant and does not effect reported associations. The sensitive assay outcome groups as percentages are 62.0 *vs* 14.5 *vs* 13.0 *vs* 10.5 for TS *vs* DIR *vs* DVR *vs* TF respectively.

The baseline characteristics of the group as a whole and the four treatment outcomes are shown in Table 3.3. Patients experiencing TF were significantly younger compared to TS and DIR patients. Over time there has been a

significant increased trend in the number and proportion of women commencing cART (7.8% women in 1996 *vs* 12.7% in 2002; $\chi^2$ for trend p-value 0.011). Similarly the proportion of heterosexuals also increased (9.1% in 1996 *vs* 23.3% in 2002, $\chi^2$ for trend p-value 0.003). There was no difference in the proportion of intravenous drug users (IDU) commencing cART over time, but they were significantly more likely to experience treatment failure. Patients with TS and DVR had lower baseline CD4 T-cell counts and patients with DVR had higher baseline VL. Patients with DIR had lower baseline VL. After 12 months of therapy the median CD4+ T-lymphocyte count was 328 cells/μL, with a median change of +140 cells ($p<0.001$). The rise in CD4 cells was significantly higher in TS *vs* DVR ($p=0.003$) and the fall was significantly greater in TF *vs* DIR. Eight hundred and fifty-five (74.9%) patients experienced a rise of at least 50 CD4 T-lymphocytes.

Of the 198 patients with a detectable VL at 12 months, 113 (57.1%) had a VLBLD at least once in the initial 12 months of therapy, 68 DVR and 45 TF. Of the patients with a detectable virus at 12 months, 53 (27%) had changed their therapy, 22 DVR and 31 TF. The 12 month VL was significantly higher in TF *vs* DVR, $p<0.001$. Of all patients, 1056 (92.6%) had had a VLBLD at some point during the first 12 months of therapy, treating excluded patients equals failure, 77% had ever had a VLBLD.

**Table 3.3: Baseline characteristics and CD4 T-cell count and viral load after 12 months on cART**

| Quadrant number (%) | Mean age years (SD) [95% CI] | Male number (%) | Transmission Risk number (%) | | | CD4 T-cell count (cells/µL) median (IQR) | Viral load (copies/ml) median (IQR) | 12 months on cART | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | MSM | HET | IDU | | | CD4 T-cell count (cells/µL) median (IQR) | Change in CD4 T-cell count | Viral load (copies/ml) | Sensitive assay number viral load <50 copies/ml/ number tested (%in quadrant of total tested (904)) |
| All 1141 | 37.6 (8.4) | 984 (86.2) | 843 (73.9) | 233 (20.5) | 68 (5.9) | 175 (76-292) | 71,138 (16,739-235,193) | 328 (209-471) | +140 (51-243) | <500 943 (82.8%) | 758/904 (82.7) |
| TS 757 (66.4) | 37.6 (8.3) [37.0 - 38.2] | 647(65.8) | 550 (65.2) | 166 (71.2) | 38 (55.9) | 157 (64-256) | 88,792 (22,254-285,000) | 365 (254 - 504) | +193 (123-288) | <500 757(100.0%) | 560/611 (62.0) |
| DIR 186 (16.3) | 39.5 (9.8) [38.1 - 40.9] | 168(17.1) | 146 (17.3) | 30 (12.9) | 8 (11.8) | 264 (156-407) | 29,791 (2,008-96,226) | 235 (126 - 351) | -7.5 (-52-27) | <500 186(100.0%) | 131/147 (14.5) |
| DVR 98 (8.6) | 36.4 (6.8) [35.0 - 37.8] | 83(8.4) | 72 (8.5) | 19 (8.2) | 7 (10.3) | 151 (52-268) | 101,818 (36,406-326,235) | 314 (226 - 465) | +148 (99- 210) | 3,017 (1076 - 20,969) | 0/67 (7.4) |
| TF 100 (8.8) | 35.5 (7.5) [34.0 - 37.0] | 86(8.7) | 75 (8.9) | 18 (7.7) | 15 (22.1) | 225 (102-376) | 67,091 (11,774-173,489) | 182 (76 - 299) | -23 (-93-10) | 32,672 (6,538 - 103,354) | 0/79 (8.7) |
| p-value | <0.001^ | 0.363 | 0.175 | 0.309 | <0.001 | <0.001* | <0.001* | <0.001* | <0.001* | | |

^ using one way analysis of variance
*p-value using Kruskal-Wallis test
TS = treatment success
TF = treatment failure
DIR = discordant immunological response; VLBLD and CD4 T-cell rise <50
DVR = discordant virological response; detectable viral load and CD4 T-cell rise >50
MSM = men who have sex with men
HET = heterosexual
IDU = injecting drug use
IQR = inter-quartile range

Anti-retroviral therapy is detailed in Table 3.4. Change to general clinic practice resulted in NNRTI being increasingly prescribed as first line therapy over the study period, from 2% in 1996 to 73% in 2002, and boosted PI therapy increased as a proportion of all PI based cART over the study period, from 2% to 35.7%. Six hundred and twenty-one patients (54.4%) remained on their first line cART at 12 months, 419 (36.7%) switched treatment within 1 year of commencing cART, and 101 (8.9%) had stopped therapy. There was no difference in rate of therapy switch between the groups (40 (40%) *vs* 37 (37.8%) *vs* 67 (36.0%) *vs* 275 (36.3%) for TF *vs* DVR *vs* DIR *vs* TS). More patients with detectable virus had stopped therapy 41 (41%) *vs* 21 (21.4%) *vs* 10 (5.4%) *vs* 29 (3.8%) for TF *vs* DVR *vs* DIR *vs* TS.

Patients commencing NNRTI were more likely to experience TS than those commencing PI, although this difference was lost when only considering boosted PI regimens. NNRTI patients were also less likely to have a DVR when compared to all PI patients, but not boosted PI (Table 3.4).

**Table 3.4: Baseline cART for all patients and by quadrant**

| Quadrant | All Patients number (%) [95% CI] n=1141 | NNRTI number (%) [95% CI] n=666 | PI number (%) [95% CI] n=441 | Boosted PI number (%) [95% CI] n=57 | Unboosted PI number (%) [95% CI] n=384 | Triple NA number (%) [95% CI] n=59 |
|---|---|---|---|---|---|---|
| TS | 757 (66.4) [63.6 to 69.1] | 465 (69.8) [66.3 to 73.3] | 272 (61.7) [57.1 to 66.2] | 40 (70.2) [56.6 to 81.6] | 232 (60.4) [55.5 to 65.3] | 36 (61.0) [47.4 to 73.5] |
| DIR | 186 (16.3) [14.2 to 18.4] | 113 (17.0) [14.0 to 19.8] | 66 (15.0) [11.6 to 18.3] | 7 (12.3) [5.1 to 23.7] | 59 (15.4) [11.8 to 19.0] | 11 (18.6) [9.7 to 30.9] |
| DVR | 98 (8.6) [7.0 to 10.4] | 43 (6.5) [4.7 to 8.6] | 54 (12.2) [9.2 to 15.3] | 3 (5.2) [1.1 to 14.6] | 51 (13.3) [9.9 to 16.7] | 4 (6.8) [1.9 to 16.5] |
| TF | 100 (8.8) [7.2 to 10.6] | 45 (6.8) [5.0 to 8.9] | 49 (11.1) [8.2 to 14.0] | 7 (12.3) [5.1 to 23.7] | 42 (10.9) [7.8 to 14.1] | 8 (13.6) [6.0 to 25.0] |

TS = treatment success
DIR = discordant immunological response; VLBLD and CD4 T-cell rise <50
DVR = discordant virological response; detectable viral load and CD4 T-cell rise >50
TF = treatment failure
CI = confidence interval
NA = nucleoside analogue
NNRTI = non-nucleoside reverse transcriptase inhibitor

We analysed the slope of CD4 T-cell count decline in the 24 months prior to the commencement of cART. Patients with TS and DVR had a higher rate of decline than those in other quadrants, being 9.7 and 9.5 cells/μL/month *vs* 4.8 and 4.7 for DIR and TF respectively, p<0.001 (Table 3.5, Figure 3.4). Following cART initiation, the rate of change of CD4 T-cell counts for the first 12 months was + 8.7 cells/μL/month for TS, +5.8 for DVR, +2.3 for DIR and –3.7 for TF, TS *vs* DVR p<0.001 (Figure 3.4).

**Table 3.5: Rate of decline of CD4 T-cell count for 24 months prior to the initiation of cART**

| Quadrant | Number (%) total = 1141 | Rate of decline in CD4 T-cell count cells/mm$^3$/3 months [95%CI] |
|---|---|---|
| **TS** | 757 (66.3) | 29.2 [27.4 to 30.9] |
| **DIR** | 186 (16.3) | 14.5 [11.1 to 18.0] |
| **DVR** | 98 (8.6) | 28.4 [23.8 to 32.9] |
| **TF** | 100 (8.8) | 14.1 [9.8 to 18.5] |

TS = treatment success
DIR = discordant immunological response; VLBLD and CD4 T-cell rise <50
DVR = discordant virological response; detectable viral load and CD4 T-cell rise >50
TF = treatment failure
CI = confidence interval

**Figure 3.4: Rate of CD4 T-cell count decline and recovery pre- and post-initiation of cART**

*TS=treatment success*
*DIR = discordant immunological response; VLBLD and CD4 cell rise <50*
*DVR =discordant virological response; detectable viral load and CD4 cell rise >50*
*TF=treatment failure*

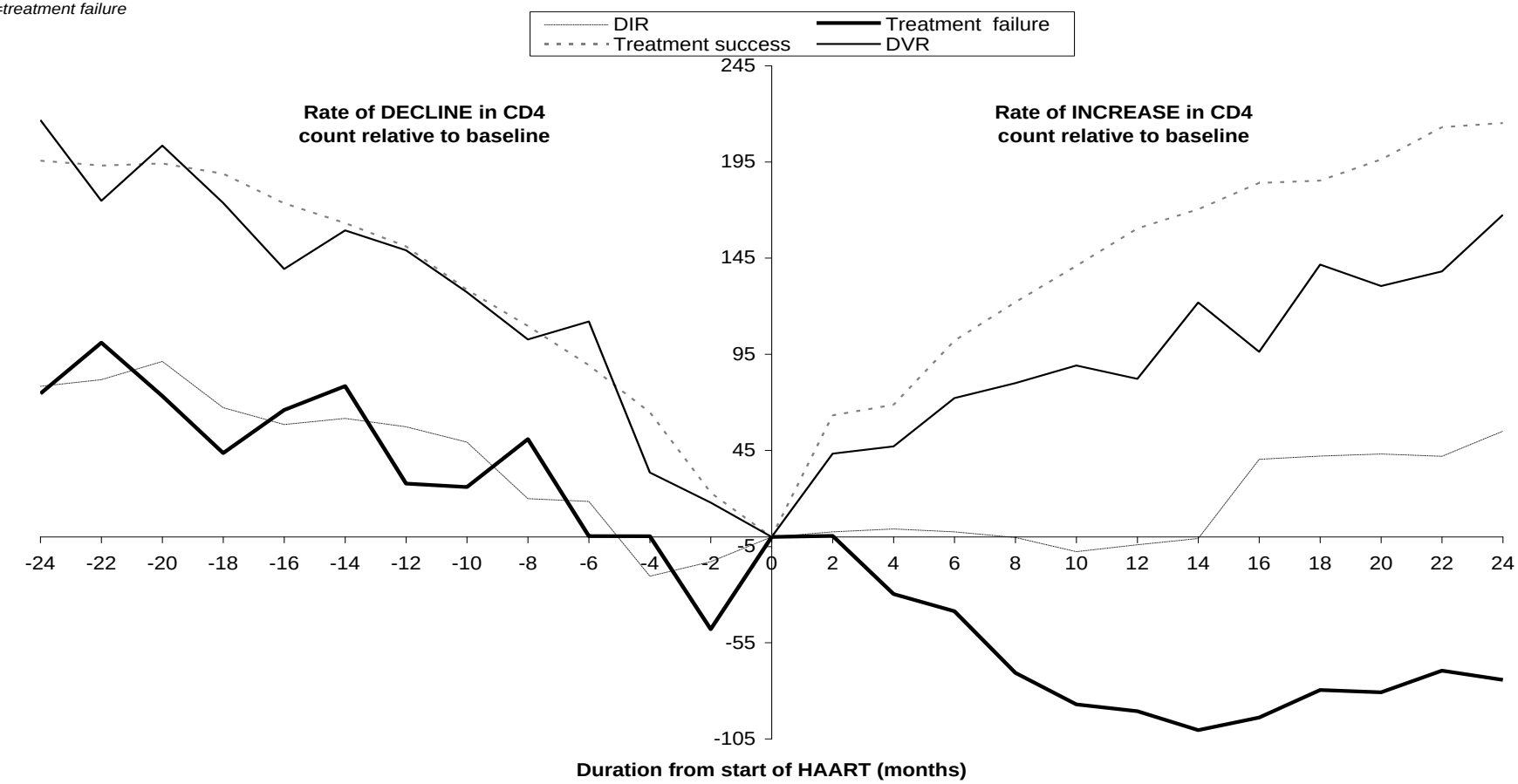


Table 3.6 shows the disposition of patients (data available for 755) at 24 months, using the same definitions of outcome. Of TS patients 87.9% maintained this to 24 months. Patients with a DIR were significantly more likely to move to TS than TF. Of DVR patients, 50% achieved a VLBLD by 24 months, and 44.8% TF achieved a VLBLD by 24 months. At 24 months the number of patients with TS had significantly increased to 72.6%, largely from a decreasing number with DIR (11.3%).

**Table 3.6: Treatment outcome at 24 months relative to quadrant at 12 months**

| Quadrant at 12 months for patients with 24 month data n=755 number (%) | Quadrant at 24 months | Number (%) [95% CI] | Quadrant at 24 months number n=755 number (%) [95% CI] |
|---|---|---|---|
| TS 505 (66.9) | TS | 444 (87.9) [85.1 to 90.8] | TS 548 (72.6) [69.4-75.8] |
| | DIR | 17 (3.4) [2.0 to 5.3] | |
| | DVR | 30 (5.9) [4.1 to 8.4] | |
| | TF | 14 (2.8) [1.5 to 4.6] | |
| DIR 122 (16.2) | TS | 57 (46.7) [37.9 to 55.6] | DIR 85 (11.3) [9.0-13.5] |
| | DIR | 54 (44.3) [35.4 to 53.1] | |
| | DVR | 4 (3.3) [0.9 to 8.2] | |
| | TF | 7 (3.3) [2.3 to 11.5] | |
| DVR 70 (9.3) | TS | 34 (48.6) [36.4 to 60.8] | DVR 65 (8.6) [6.7-10.8] |
| | DIR | 1 (1.4) [0.03 to 7.7] | |
| | DVR | 26 (37.1) [25.9 to 49.5] | |
| | TF | 9 (12.9) [6.1 to 23.0] | |
| TF 58 (7.7) | TS | 13 (22.4) [12.5 to 35.3] | TF 57 (7.5) [5.8-9.7] |
| | DIR | 13 (22.4) [12.5 to 35.3] | |
| | DVR | 5 (8.6) [2.9 to 19.0] | |
| | TF | 27 (46.6) [33.3 to 60.1] | |

TS = treatment success
DIR = discordant immunological response; VLBLD and CD4 T-cell rise <50
DVR = discordant virological response; detectable viral load and CD4 T-cell rise >50
TF = treatment failure
CI = confidence interval

Seventy-two patients (6.3%) experienced disease progression (DP), becoming symptomatic (non-AIDS) or having an AIDS defining illness, during the initial 12 months of cART. Patients who experienced a rise in their CD4 T-cell count experienced less DP (TS 5.3% [95%CI 3.8-7.1] and DVR 6.1% [2.3-

12.8]) than patients without a CD4 T-cell rise (DIR 9.1% [5.4-14.2] and TF 9.0% [4.2-16.4]); TS *vs* DIR p= 0.048. Sixteen patients died between 12 and 24 months. TS patients (6 (1.1%) [95% CI- 0.4-2.4] were less likely to die *vs* TF (7 (12.3%) [5.1-23.7]). No DVR patients died, [0.0-5.5, using exact method] and 3 DIR ((3.5%) [0.7-10.0]) patients had died by 24 months. Twenty-four patients experienced disease progression or death by 24 months, none in DVR (0%, [0-5.1]), 11 TS (2.2%, [1.1-3.9]), 6 DIR (4.9%, [1.8-10.4]) and 7 TF (12.1%, [5.0-23.3]).

*3.3.5 Discussion*

Discordant responses to cART in ARV naive HIV-1 infected individuals are not uncommon phenomena. The main differences observed between studies are largely due to different definition criteria for response, varying assessment time periods and previous therapy which may influence outcomes.

TS occurred in 66.4% of our cohort, which is higher than that seen in other studies, largely due to their shorter follow-up periods and inclusion of ARVT experienced patients (Picketty *et al*., 1998, Perrin *et al*., 1998, Barreiro *et al*., 1999, Grabar *et al*., 2000).

DIR was observed in 16.3% of patients at 12 months, compared to a wide range reported elsewhere; 5 -29% (Picketty *et al*., 1998, Perrin *et al*., 1998, Pakker *et al*., 1999, Renaud *et al*., 1999, Barreiro *et al*., 1999, Grabar *et al*., 2000). The reasons for a DIR are likely to be multiple and complex. Ongoing low level viral replication, either below the level of detection or occurring intermittently and thus not captured, could lead to chronic low level activation of the immune system. This may either be augmented by, or be primarily due to the translocation of foreign proteins across gut mucosa due to the HIV-1 related depletion of GALT. The resulting increase in CD4 T-cell turnover (Garcia *et al.*, 2000, Anthony *et al.*, 2003) could limit the CD4 T-cell

recovery. This increase in CD4 T-cell turnover is seen in ARV naïve individuals and decreases with the introduction of cART, although it may not normalise (Anthony *et al.*, 2003). Individuals with a slower decline in T-cell turnover in response to cART initiation may be those who take longer to achieve a TS; those initially categorised as DIR who become TS by month 24. Furthermore it is possible the ongoing immune system activation is driven by compartmentalized viral antigens, for example in germinal centres (Orenstein *et al.*, 1999). This dissociation between VL and CD4 T-cell turnover and recovery has been demonstrated by Leng and colleagues (Leng *et al*., 2001) who showed that CD4 T-cell counts correlated more strongly with activation and turnover than with VL. More sensitive viral load assays and measurements of pro-viral DNA have shown an inverse correlation with CD4 T-cell counts (Chun *et al.*, 2002). This supports the concept that low level undetectable ongoing viral replication drives immune activation and limits CD4 T-cell recovery. This hypothesis has clinical implications for the types of monitoring employed in the management of HIV-1 infected individuals and the treatment strategies which may need to be employed to address DIR. This may be via intensification or by utilising specific ARVT as there is some evidence that PI based regimens may impact on the CD4 T-cell recovery in response to the initiation of cART (Smith *et al.*, 2002). It also suggests a potential role for immunosuppressive therapy in certain patients.

Previously reported risk factors for DIR include older age (Lederman *et al*., 2000, Teixeira *et al*., 2001, Viard *et al*., 2001, Yamashita *et al*., 2001, Florence *et al.*, 2003), HIV-1 transmission group (Viard *et al*., 2001) and lower baseline CD4 T-cell count and VL (Grabar *et al.*, 2000, Florence *et al.*, 2003). We did not observe any association between DIR and older age or transmission risk group, although a trend was seen with age. This association is thought to be

related to the observed decrease in thymic function seen with age (Douek *et al.,* 1998). In contrast to the EuroSIDA data we observed an association between DIR and a higher baseline CD4 T-cell count, but a similar association with lower baseline VL. The differences observed may be due to the EuroSIDA study only including those with a baseline CD4 T-cell count <300/μL and then comparison being made within those achieving VLBLD, it also included ARV experienced patients, albeit not optimal cART.

It is possible ARV therapy may, in some way, be damaging to CD4 T-cells, limiting their capacity to regenerate, and several *in vitro* studies have demonstrated toxicity (Viora *et al.*, 1994, 1997, Benveniste *et al.*, 2001). However we did not find any difference in outcome group between the different classes of drugs. Florence and colleagues did demonstrate a potential advantage in patients on triple class therapy suggesting a role for maximal virological suppression, however the numbers were small and the confidence intervals wide (van Praag *et al.*, 2002, Florence *et al.*, 2003).

DVR was seen in 8.6% comparable to the 7-19% reported elsewhere (Barreiro et al., 1998, Piketty et al., 1998, Kaufmann *et al.*, 1998, Levitz, 1998, Grabar et al., 2000, Fessel *et al.*, 2000). It is likely the majority of these patients actually achieved a VLBLD at time points prior to the 12 month time point, thus allowing some degree of immune recovery. We observed that 69.4% (68/98) of patients with DVR had at least one recorded undetectable VL in the first 12 months of therapy. Additionally the VL at 12M was significantly lower than that in the TF group, making it likely that there was a lower level of immune activation and hence the potential for more immune recovery. Other factors such as viral pathogenicity and ARV drug effects are likely to play a role in the immune system being able to recover to a degree in the face of ongoing viral replication. This may occur with selection of a mutant virus

which is less fit compared to wild type virus (Hejdeman *et al.*, 2001). It has also been reported that PI therapy positively effects CD4 T-cell apoptosis independent of their antiviral effect (Sloand *et al.*, 1999, Chavan *et al.*, 2001)

TF occurred in 8.8% of patients which is less then other studies (Grabar *et al.*, 2000). Younger patients and those with IVDU as their HIV transmission risk category were more likely to experience TF.

Overall, 83% of patients achieved an undetectable VL and this is consistent with outcomes observed in ARV therapy trials. A total of 74.9% achieved significant recovery of their CD4 cell count and this would appear to confer a degree of protection from disease progression regardless of the accompanying virological response. Other studies have also reported a superior effect on clinical outcome of an immunological response compared to, and independent of, a virological response (Grabar *et al.*, 2000). It may be this reflects the relative level of immune activation present in the individual; those with an immunological response even with a detectable VL have a relatively less activated immune system, conferring benefit. However the best clinical outcomes are observed in those experiencing TS (O'Brien *et al.*, 1997, Marschner *et al.*, 1998).

Examining the CD4 T-cell slopes before and after the initiation of cART reveals a significant difference between immunological responders (IR; TS and DVR) compared to immunological non-responders (INR; TF and DIR); the former experiencing more rapid decline and recovery of their CD4 T-cell count. The more rapid decline is consistent with the higher VL and lower CD4 T-cell count seen at baseline in IR. The steeper slope is likely to be a reflection of higher levels of immune activation and cell turnover. Once the stimulus to this is removed by initiating cART (even if removal is incomplete as in DVR) this group manages to recover immunologically at a faster rate compared

to INR. This may be due to maintenance of the previously high cell production without the previously high rate of viral related cell death due to the effect of cART. It is unclear what factors will decide whether a patient is an IR or an INR but it is likely both individual and viral factors will play a role. Although not demonstrated in this cohort study, age has been shown to be associated with DIR (Lederman *et al*., 2000, Teixeira *et al*., 2001, Florence *et al*., 2003) and is known to influence thymic T-cell production (Douek *et al*., 1998). Another factor may be the presence of lymphoid tissue fibrosis impacting on CD4+ T-cell recovery. This fibrosis occurs as a consequence of immune activation and leads to depletion of naïve T–cells by restricting access to the fibroblastic reticular cell (FRC) network and the associated T-cell survival factor IL-7. Compounding this is the consequent decreased production of the FRC survival factor, lymphotoxin-β by T-cells, setting up a vicious cycle of T-cell depletion and FRC network loss (Estes, 2009, Zeng *et al*., 2011).

Identification and characterisation of such factors is important given the effect this has on the clinical outcome. Modelling such patterns may enable prediction of treatment outcome based on the slope of an individual's pre-treatment CD4 T-cell decline, potentially allowing interventions and monitoring to address potential problems.

By 24 months a significant number of patients had changed their outcome group, in many cases reflecting a delayed response to treatment, with DIR becoming TS and TF achieving a VLBLD. The vast majority of these moves were beneficial in terms of treatment outcome. This apparent delay has also been observed in other studies (Grabar *et al.*, 2000). However as there appears to be a clinical consequence of the primary treatment outcome

group assignment, this observation requires more prospective long term outcome studies.

The changing demographics observed in this cohort over time, with increasing proportions of women and heterosexuals commencing cART, reflects the changing face of the UK epidemic (HPA) and the local clinic population. Ethnic differences have been observed in levels of immune system activation but no difference was seen in outcome in this study, however the numbers are small.

The large number of patients and the long follow up period are strengths of this study. One limitation is its retrospective cohort study design with the inherent associated biases. A further limitation is the number of ineligible patients due to missing data (n=225, 16%) which may introduce bias with some groups, such as IVDU and poor treatment responders, being potentially more likely to be lost to follow up or inconsistent attenders.

In summary, discordant responses are frequent following the initiation of cART. In DIR the role of low level viral replication and immune activation may explain the blunted immune recovery observed in some individuals. It will be important to delineate this phenomenon further as it impacts negatively on clinical outcome despite the apparent virological success of the regimen. It suggests more sensitive monitoring and treatment intensification may be required. Furthermore it raises the possibility of immune therapy, for example with IL-2, to address this deficit in the immune system's capacity to regenerate. A further option worth investigating would be therapy directed towards the ongoing immune system activation with immuno-suppressants such as hydroxylchloroquine. DVR occurs less frequently and may largely be a function of timing of the VL measurement. In the small number of individuals it affects it may be influenced by the class of ARV therapy, and

whilst no difference was observed in this study between classes, possibly due to small numbers, this warrants further investigation given the current *in vitro* data.

## 3.4 Study Two: Restoration of HIV-1-specific responses in patients changing from protease to non-nucleoside reverse transcriptase inhibitor based antiretroviral therapy. (Reproduced, with minor updated changes, as published, see Chapter 8).

### *3.4.1 Introduction*

A major improvement in the morbidity and mortality associated with HIV-1 disease has occurred as a result of the introduction of combination antiretroviral therapy (cART) (Egger *et al.* 1997, Hammer *et al.*, 1997, Camerone *et al.*, 1998, Palella *et al.*, 1998, Detels *et al.*, 1998). This treatment typically involves a two nucleoside analogue (NA) backbone to which is added either a NNRTI or a PI. Consensus guidelines recommend NNRTI based regimens as initial therapy based on easier adherence and more favourable toxicity profiles in the context of surrogate marker equivalence (Gazzard *et a*l., 2008).

Combinations containing a PI have been associated with improved clinical outcome (Egger *et al.* 1997, Hammer *et al.*, 1997, Palella *et al.*, 1998, Detels *et al.*, 1998, Camerone *et al.*, 1998), and a degree of immune reconstitution (*Arno et al.*, 1998, Pakker *et al.* 1998, Zhang *et al.*, 1998), enabling discontinuation of primary and secondary prophylaxis against opportunistic infections (Gill *et al.*, 1998, Furrer *et al.*, 1999, Jouan *et al.*, 1999, El-Sadr *et al.*, 2000,). Regression of Kaposi's sarcoma has also been reported (Lebbe *et al.*, 1998, Martinelli *et al.*, 1998). Some of the disadvantages of early PI regimens

included relatively higher frequency of administration, higher pill burden, medication refrigeration requirements, the development of metabolic abnormalities and increased risk of long term complications such as cardiovascular disease (Eastone *et al.*, 1997, Carr *et al.*, 1998, Henry *et al.*, 1998, Sullivan *et al.* 1998, Carr *et al.*, 2000, Bonnet *et al.*, 2000).

Regimens containing NNRTI have been shown to provide at least equivalence in improvements in the surrogate markers of HIV-1 infection when compared with a PI containing regimen (Staszewski *et al.*, 1999). NNRTI regimens tend to be easier to adhere to, a key factor in the success of cART (Lucas *et al.*1999), and have fewer known associated serious toxicities. One potential disadvantage of a NNRTI containing regimen is the genetic barrier to the development of drug resistance for most drugs in this class, whereby a single mutation in the reverse transcriptase gene of HIV-1 is associated with marked reduction in sensitivity to the NNRTI and potentially cross resistance to other drugs within that class (Condra *et al.*, 1992, Richman *et al.*, 1994, Havlir *et al.*, 1996). Another potential disadvantage of the NNRTI containing regimens is the paucity of data on improvements of immune function in individuals with advanced HIV-1 infection. In studies with predominantly PI containing regimens, improved lymphocyte proliferative responses (LPR) to mitogens and a variety of recall antigens have been shown to occur within months of initiating therapy. Increases in HIV-1-specific proliferative responses have been demonstrated in early HIV-1 disease (Rosenberg *et al.*, 1997, Al-Harthi *et al.*, 2000), but have not been observed in more advanced disease (Kelleher *et al.*, 1996, Autran *et al.*, 1997, Li *et al.*, 1998). Whether this improvement is due to proliferation of pre-existing CD4+ and CD8+ memory T-cells or the generation of new naïve CD4+ and CD8+ T-cells is unclear. Moderate improvements in the numbers of naïve cells do occur in response

to therapy (Li *et al.*, 1998), and techniques to assess thymic function suggest that these are derived from the thymus (Douek *et al.*, 1998).

HIV-1 infected individuals taking a PI containing regimen may wish to switch to treatment containing a NNRTI either to avoid toxicity or to enhance the convenience of the regimen (Gatell, 1998, Murphy *et al.*, 2002). This appears to be a safe treatment intervention in respect to ongoing virological control (Martinez *et al.*, 1999, Raffi *et al.*, 2000, Barreiro *et al.*, 2000). Several reports (Andre *et al.*, 1998, Chavan *et al.*, 2001), have suggested an adverse effect of PI on antigen presentation and cell activation. We have also observed a different class effect of these drugs on the levels of the β-chemokines MIP-1a and MIP-1β (Burton *et al.*, 2002). These observations prompted us to investigate possible different effects the two drug classes have on the functional immune reconstitution within such a patient population. Such data would potentially provide reassurance that NNRTI containing regimens provided similar capability of immune reconstitution as PI. We have therefore prospectively studied 8 HIV-1 infected patients switching from a PI to a NNRTI containing regimen to assess the immunological responses present in such individuals before and after switching therapy.

*3.4.2 Materials and Methods*

3.4.2.1 Patients

Seven patients with long term negative viral loads were prospectively evaluated when switching from a PI to an efavirenz (EFV, DuPont, Pharma, Stevenage, UK) containing regimen; the regimen's NA backbone was unchanged for all patients. One patient switching to an efavirenz containing regimen because of virological failure was also studied. Patients had blood samples drawn at baseline and at 12 and 24 weeks following the change of

therapy. The study had Local Research Ethics Committee approval and all patients provided written informed consent.

3.4.2.2 Plasma viral RNA assay

Viral load in patient plasma was measured at each time point of sample collection using the Quantiplex HIV RNA 3.0 (Chiron bDNA) assay (detection limit <50 copies/ml, Chiron Diagnostics, Halstead, UK).

3.4.2.3 Antibodies and Flow cytometry

The following murine monoclonal antibodies (mAb) were used: anti-human CD3, CD4, CD8, CD19 and CD56 (TetraOne, Beckman Coulter, High Wycombe, UK). The Epics XL-MCL (Beckman Coulter) was used for flow cytometric analyses of total $CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$ and $CD56^+$ lymphocytes using whole blood.

3.4.2.4 Isolation of PBMC and Culture Conditions

PBMC were isolated by density gradient centrifugation and cultured fresh in supplemented RPMI-1640 medium (Imami *et al.*, 1994, Imami *et al.*, 1999). All cell lines were cultured in 10% FCS/RPMI in 25 $cm^2$ flasks (Greiner Laboritechnik, Dursley, UK) and incubated at 37°C.

3.4.2.5 Recombinant HIV-1 antigens

These were obtained from Medical Research Council Centralised Facility for AIDS Reagents (NIBSC, Potters Bar, UK).

3.4.2.6 Proliferation assays

PBMC ($10^5$/well) were cultured with antigen, mitogen or cytokine in round bottomed microtiter plates (Greiner), in 10% AB plasma/RPMI (200 µl; Sigma). Antigens and mitogens were used at concentrations previously described (Imami *et al.*, 1999). In addition toxoplasma antigen (Behring, Milton Keynes, UK) at a final concentration of 10 µg/ml was added. IL-2 was used in this study at 20U/ml and 100U/ml (Roche Diagnostics, Welwyn Garden City, UK).

On day 5, 100 µl of supernatant was collected and stored at -20°C for subsequent cytokine measurement. Each well was then pulsed with 1 µCi $^{3}$H-methyl thymidine ($^{3}$H-TdR; Amersham International, Amersham, UK) and 16 h later cells were harvested onto glass fibre filtermats (Wallac Oy, Turka, Finland). Proliferation as measured by $^{3}$H-TdR incorporation was evaluated by liquid scintillation spectroscopy using a 1205 Betaplate counter (Wallac) (Fessel *et al.*, 2000, Imami *et al.*, 2001). Results are expressed as stimulation index (SI) and as mean counts per minute (cpm) for triplicate cultures, with percentage error of the mean <15%. A positive response is defined as a SI of five or more. Control wells, for calculation of background activity, contained PBMC only.

3.4.2.7 Measurement of IL-2 and IL-4 Production

For cytokine assays, 50 µl of supernatant from proliferative cultures, were transferred in two sets of 96-well round bottomed plates as triplicates for measurement of IL-2 and IL-4 using indicator cell lines CTLL-2 (European Collection of Animal Cell Culture, Salisbury, UK; ECACC) and CT.h4S (a generous gift of W. Paul, Bethesda, MD) as described previously (Fessel *et al.*, 2000, Imami *et al.*, 2001). Briefly, CTLL-2 ($10^{3}$ cells/well) or CT.h4S ($5x10^{3}$ cells/well) were added in 50 µl to give a final 100 µl. After 24 h in culture, wells were pulsed with $^{3}$H-TdR and harvested as described above.

3.4.2.8 Latent proviral DNA

HIV-1 proviral DNA was measured utilising spectophotometric PCR methodology (Hardy *et al.*, 1999), with an analytic sensitivity of 10 copies/µg of total cellular DNA.

3.4.2.9 Statistical analysis

Data are presented as proportions, and where appropriate 95% confidence intervals are also presented which are derived from the binomial distribution using the exact method. Within patient variability over time was assessed using the repeated measures analysis of variance. Any variability between patients was assumed to be random.

*3.4.3 Results*

Eight patients previously treated with a PI containing regimen (mean treatment time 20 months, range 4-33 months, 7 patients $\geq$14 months) were included in this study. In seven patients the plasma HIV-1 RNA load had been consistently undetectable using a sensitive assay (measuring less than 50 copies per ml) for a mean of 15.3 months (range 3-28 months). The eighth patient switched therapy because of an increasing viral load. In all patients the HIV-1 RNA load was measured on the day of switch. The patients took efavirenz 600 mg and their PI morning dose on the day the sample was taken. In two of the seven patients (patients 1 and 2) whose viral load had been persistently negative prior to the switch, virus was detectable in this sample (Table 3.7). In one case (patient 1) the virus could not be detected in any subsequent sample taken two monthly for ten months. In the other case (patient 2) low level viraemia (viral load less than 1,000 copies per ml) was consistently present at nine months. Over the 24 weeks following therapy switch the mean CD4 T-cell count increased from 346 to 395 cells/μl (p= not significant). This was reflected in all individuals apart from patient 4, however by week 36 his CD4 T-cell count had also risen (531 cells/μl). The rate of rise was similar for the six months prior to the switch to the NNRTI and for the six months following this (p=0.06, using repeated measures analysis of variance).

**Table 3.7: CD4 T-cell count and viral load in patients at baseline (BL) and 12 and 24 weeks (W)**

| | | CD 4 T-cell count (cells/μL blood) | | | | Plasma viral load (copies/ml) | | |
|---|---|---|---|---|---|---|---|---|
| **Pt.** | **Duration of PI therapy (months)** | **Pre-PI** | **BL** | **12 W** | **24 W** | **BL** | **12 W** | **24 W** |
| **1** | 4 | 23 | 41 | 205 | 287 | 1116 | BLD | BLD |
| **2** | 26 | 403 | 418 | 622 | 665 | 6794 | 753 | 394 |
| **3** | 14 | 297 | 243 | 214 | 267 | 55068 | BLD | BLD |
| **4** | 20 | 376 | 503 | 449 | 373 | BLD | BLD | BLD |
| **5** | 14 | 88 | 256 | 190 | 306 | BLD | BLD | BLD |
| **6** | 33 | 238 | 185 | 269 | 283 | BLD | BLD | BLD |
| **7** | 28 | 90 | 301 | 324 | 321 | BLD | BLD | BLD |
| **8** | 22 | 339 | 984 | 824 | 1005 | BLD | BLD | BLD |

BLD = below the level of detection

At baseline only one patient demonstrated a proliferative response to "low level" IL-2 (20 U/ml) stimulation (Figure 3.3A) but high level IL-2 (100 U/ml) did result in strong cellular proliferation (Figure 3.3B). Seven individuals subsequently showed a strong proliferative response to low concentration IL-2 which had not been present in six whilst on the PI (Figure 3.3A) (analysis of trends p=0.1). Responses to high concentration IL-2 remained high in all patients and were comparable to those seen before the switch (p=0.5) (Figure 3.3B).

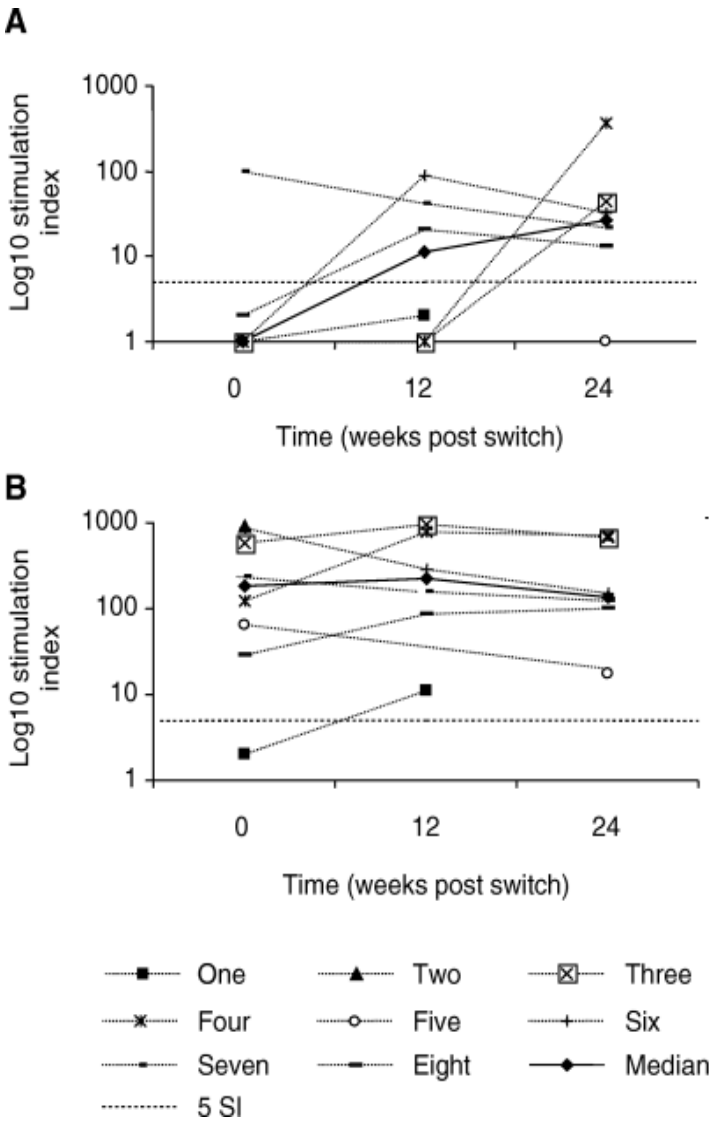


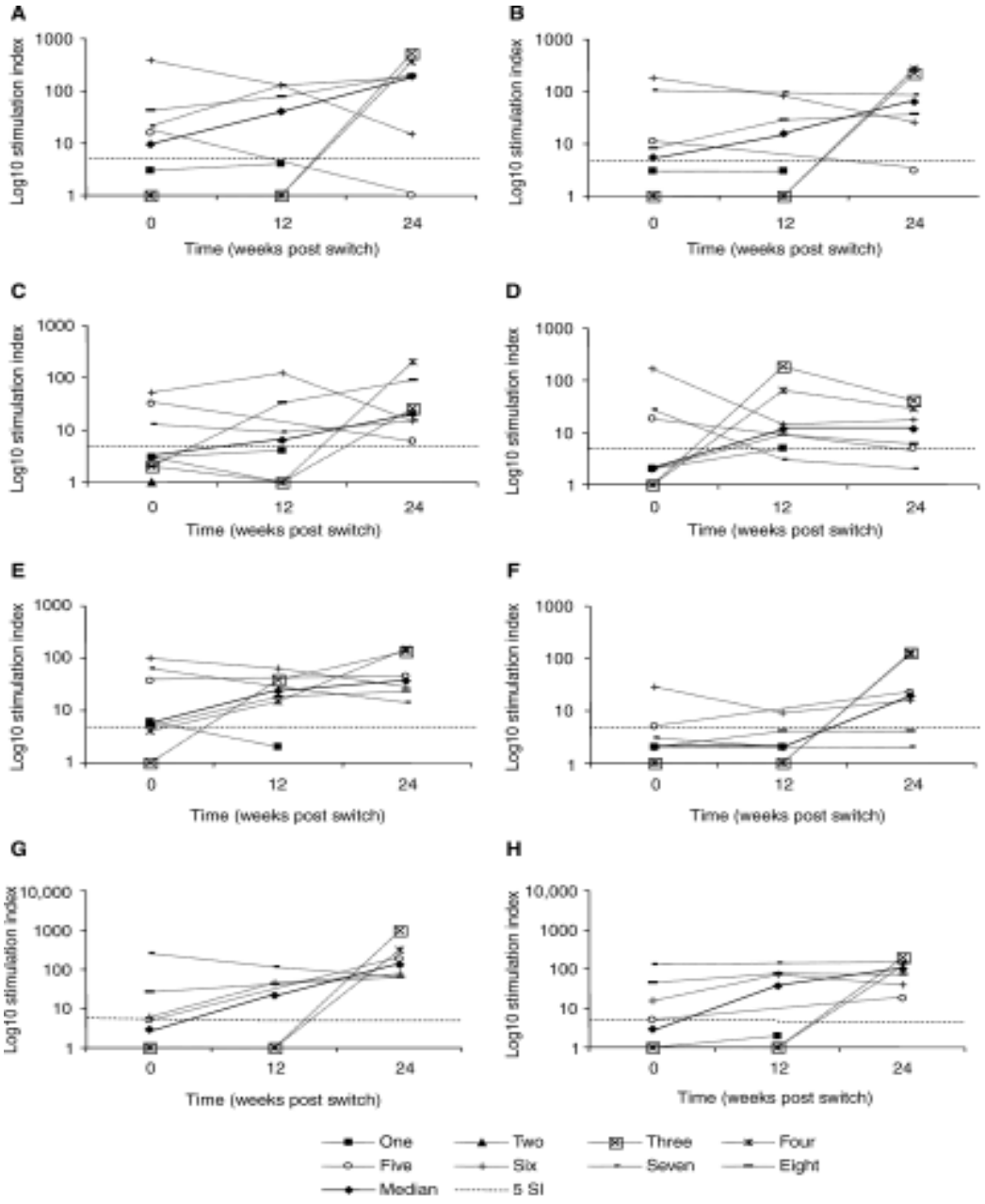

**Figure 3.5: Lymphoproliferative responses to low (20 U/ml) concentration (A) and high (100 U/ml) concentration; (B) interleukin-2 (IL-2).** Peripheral blood mononuclear cells (PBMCs) were incubated with the appropriate concentration of IL-2 for six days and $^{3}$H-thymidine incorporation was measured as described in *Materials and methods*. Results for individual patents (1–8) are expressed as stimulation index (SI) on a log scale. A positive response is defined as a SI ≥ 5.

In baseline samples while on PI, low level proliferative responses were obtained following mitogen stimulation or by the use of viral or recall antigens (Figure 3.4). Although there was some variation in individual patterns the median SI indicate similar changes in the restoration of responses for mitogens and viral and recall antigens for the cohort. Increases in HSV- and Flu-specific CD4+ T cell responses were seen, CMV-specific responses were seen in 7/8 individuals who were CMV+, whilst VZV-specific responses remained marginal (Figure 3.4A, B, C and D respectively). Equally,

an increase in response to Candida antigen was seen during follow up (Figure 3.4E), a slight improvement in responses to PPD was also seen (Figure 3.4F), whilst responses to both TTOX and Toxoplasma remained undetectable (data not shown).

Following a switch to efavirenz, significant enhancement in proliferation in response to recall and viral antigens occurred (31% increase (95% confidence interval (CI) 15%-48%)). Mitogen responses were significantly enhanced (33%, 95% CI 3-33%) in response to a switch to NNRTI therapy. Such responses were present by three months and significantly enhanced by six months for mitogens (analysis of trends PWM p=0.05, CON A p=0.03, PHA p=0.07) and PPD (p=0.06). Although there were significant increases in median SI for individual antigens overtime, analysis of trends revealed that p values were not significant for the remaining viral and recall antigens.

**Figure 3.6: Lymphoproliferative responses to viral and recall antigens and mitogens.** Peripheral blood mononuclear cells (PBMCs) were incubated with herpes simplex virus (HSV) (A), influenza A (FLU) (B), cytomegalovirus (CMV) (C), varicella zoster (VZV) (D), candida (CAND) (E), purified protein derivative (PPD) (F), concanavalin A (CON A) (G) and pokeweed mitogen (PWM) (H) antigens for six days and $^{3}$H-thymidine incorporation was measured as described in *Materials and methods*. Results for individual patients (1–8) are expressed as stimulation index (SI) on a log scale. A positive response is defined as a SI ≥ 5. No patient experienced symptomatic disease of or received vaccination for any of these agents.



The median LPR to each of the three HIV-1-specific recombinant antigens passed the positive SI threshold of five in response to the therapy change (Figure 3.7). Different patterns of change were observed in individual patients, but median SI values for the group increased from <5 to >5 for all three antigens, and the proportion of patients developing a positive response also increased. Whilst responses to both gp120 and *nef* recombinant antigens were further increased at 6 months after therapy change, responses to the capsid protein p24 plateaued, but were nevertheless sustained over the 6 month period (analysis of trends gp120 p=0.2 p24 p=0.4 and nef p=0.4).

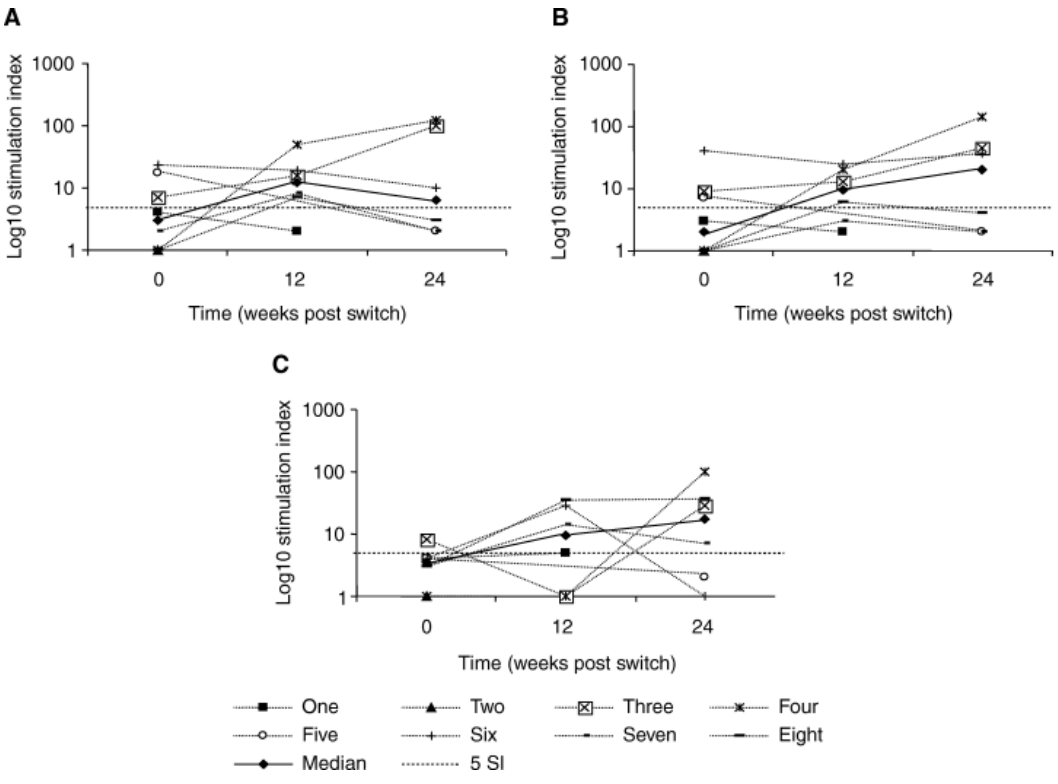



**Figure 3.7: Lymphoproliferative responses to HIV-1-specific recombinant antigens.** Peripheral blood mononuclear cells (PBMCs) were incubated with p24, (A) gp120, (B) and nef (C) recombinant antigens for six days and $^{3}$H-thymidine incorporation was measured as described in *Materials and methods*. Results for individual patients (1–8) are expressed as stimulation index (SI) on a log scale. A positive response is defined as a SI ≥ 5.

Whilst IL-2 was readily detected in all cultures where significant proliferation occurred, IL-4 remained undetectable throughout. Median proviral DNA did not change following the therapy switch; 25 copies/μg of total DNA prior to switch and 26.5 copies/μg of total DNA 24 weeks following switch. Following therapy switch proviral DNA levels reflected the presence of detectable plasma virus at baseline or subsequent weeks (Figures 3.6A and B respectively).

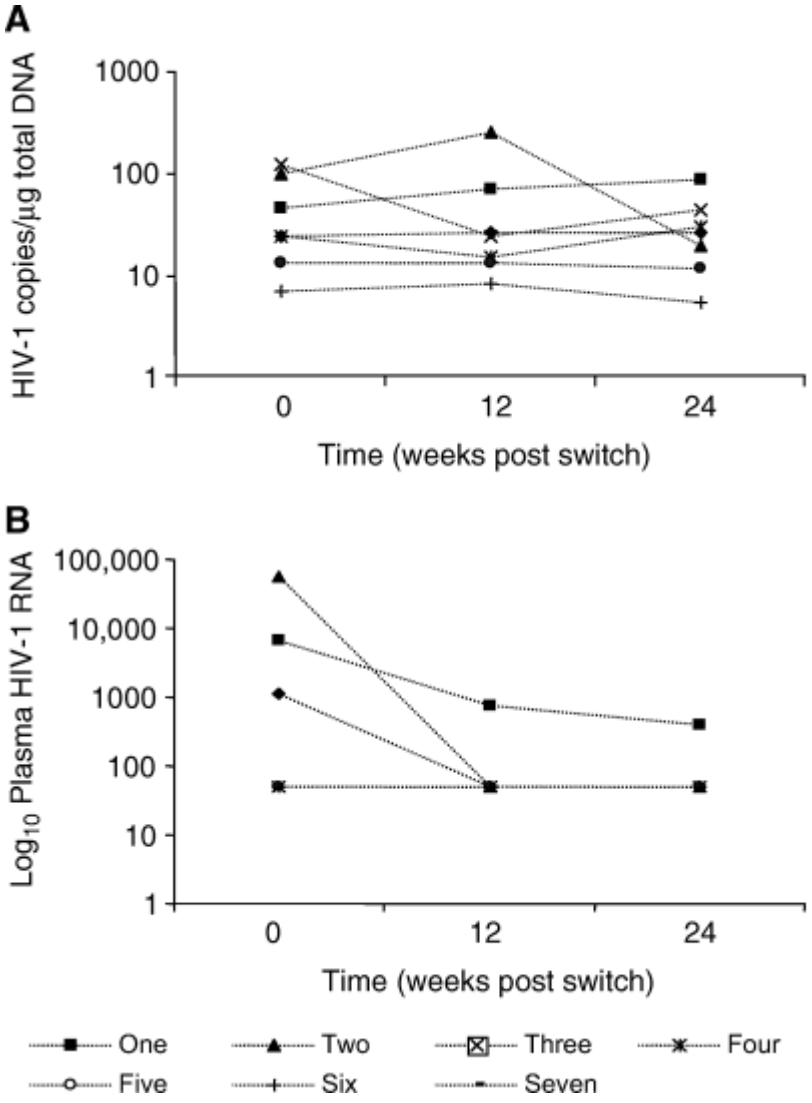



**Figure 3.8: Human immunodeficiency virus (HIV)-1 proviral deoxyribonucleic acid (DNA) (A) and plasma viral load (B) at baseline and weeks 12 and 24 after therapy switch.** Plasma HIV-1 RNA measured by bDNA assay with lower limit of detection 50 copies/ml. HIV-1 proviral DNA measured by sphectophotometric polymerase chain reaction (PCR) with an analytic sensitivity of 10 copies/μg of total cellular DNA.

*3.4.4 Discussion*

Over a six month follow up, switching from a PI to a NNRTI containing regimen appears to have been successful in seven of eight patients with sustained suppression of HIV-1 viraemia and a continuing rise in CD4 T-cell count. In two individuals a transient viraemia did occur at the time of the switch raising the possibility of adherence difficulties inherent in such a management strategy. Additionally, further studies including plasma levels of drug over the short period of the switch would be helpful to decide the optimum way in which these drugs should be administered. Despite the blip in plasma HIV-1 RNA load, proviral DNA studies revealed no alteration in DNA copies of virus per total cellular DNA.

The lower level proliferative responses in those individuals treated with regimens containing PI compared to those observed in seronegative controls are similar to those reported in other studies and in our previous experience (Kelleher *et al.*, 1996, Li *et al.*, 1998, Imami *et al.*, 2001). Improvements in these responses on switching to a NNRTI were significant and progressive. These were not random variations as they occurred across three out of four of the viral and two out of four of the recall antigens measured, most importantly including those against HIV-1, although negative for tetanus toxoid and toxoplasma. They were also accompanied by increased proliferative responses to mitogen. Although variations in individual patterns of responses following therapy switch were observed, the median SI rose. For most stimuli the median SI increased from negative to positive over time. We have previously demonstrated that in chronically HIV-1 infected individuals proliferation in response to IL-2 does not occur at the conventional low concentration used in tissue culture but does when higher IL-2 concentrations are used (Imami *et al.*, 2001). This would suggest a defective

signalling process inhibiting a normal response to IL-2 and is of interest as this appears to be restored to normal by a switch from a PI to a NNRTI containing regimen. Data from this study concur with our previous findings that only high concentrations of IL-2 were able to induce proliferative responses, however after the therapy switch cells became responsive to low concentrations too. This recovery of low dose IL-2 responsiveness may represent qualitative immune recovery with correction of impaired signalling. This would then permit the improved responses we subsequently observed with other stimuli. Whether this would translate to clinical benefit is currently unknown. There are a number of potential reasons for the improved proliferative responses on switching. Firstly this might be continuing qualitative immunological improvement and might have occurred if individuals had not switched to a NNRTI. We believe this to be unlikely and in a large group of patients treated with PI such improvements did not occur even after 65 weeks (Hardy *et al.*, 1999). Ongoing quantitative improvement with continued increasing CD4 T-cell counts was observed, although not significant over the study period and not different from that seen in the six months prior to the therapy switch. This increase may however have been sufficient to permit the degree of recovery observed. Secondly it is possible that the transient HIV-1 viraemia which occurred at the time of switching produced an anamnestic response to other recall antigens. Again, we believe this to be unlikely since responses are also seen in patients in which blips are not seen. Such a response is believed to be evanescent but in our patients some proliferative responses were greater at six than at three months.

Induction of HIV-1-specific responses in patients on PI based cART is the rationale behind many novel immunomodulatory therapeutic interventions in chronic HIV-1 disease. However, so far even the best immunotherapies have

only been able to induce transient HIV-1-specific responses and reversal to the previous anergic state is rapid (Gotch *et al.*, 1999). Patients swapping to NNRTI based regimen appear to be able to maintain these virus-specific responses. These responses are essential for control of viral replication/infection and for targeting the viral reservoirs in resting CD4+ T-cells and within lymphoid tissues.

We believe a possible explanation for the improvement in recall antigen proliferative responses may be that such responses are inhibited by the PI. Previous *in vitro* studies have suggested that the processing and presentation of antigen in relationship to class I and class II major histocompatibility antigens is defective with PI [Andre *et al.*, 1998). Interference with cell-cycle progression by protease inhibitors with resultant concentration-dependent inhibition of lymphoproliferative responses has also been demonstrated [Chavan *et al.*, 2001].

The improved responses to HIV-1 antigens demonstrated in this study are particularly important, although the clinical significance of their restoration or maintenance has yet to be demonstrated. It is possible that at least in some patients, these could have been driven by an auto-vaccination due to transient viraemia at the time of switch. There is increasing interest in the possibility that long term control of HIV-1 infection may be possible if vigorous CD4+ helper T lymphocyte (HTL) and strong CD8+ cytotoxic T lymphocyte (CTL) responses to HIV-1 antigen can be stimulated (Gotch *et al.*,1999). Therapeutic immunisation with whole inactivated gp120-depleted immunogen (Remune) induces strong HTL and CTL responses (Moss *et al.*, 1999) and some beneficial effect on surrogate markers in a subset of patients (Turner *et al.*, 2001), but appears not to confer clinical benefit (Kahn *et al.*, 2002). These results suggest when planning clinical outcome studies to

explore interventions to induce or enhance HIV specific responses, for example therapeutic or preventative vaccine studies, the potential of the ARV therapy biasing or confounding the outcomes should be allowed for and tested.

### 3.5 Summary

cART has revolutionised the management of HIV-1 infection. However, as shown in this Chapter the resultant immune recovery is neither universal nor complete. There is significant variation observed between individuals in their response to ARVT both in terms of their virological response and quantitative immune recovery as seen in Study One, having significant prognostic implications. Underlying variations in the level of residual immune activation may play a key role in an individual's treatment outcome group. The inadequacy of the associated qualitative immune restoration is highlighted in the mortality audit; a reflection of the changing patterns in the causes of death in people infected with HIV-1. These finding suggest that although there is immune recovery, it is incomplete and leaves the individual still susceptible to life threatening conditions, particularly those that may require a higher level of immune surveillance for effective protection. It would appear that manipulation of cART can impact on qualitative immune recovery as suggested in Study Two, with individuals demonstrating a benefit in switching between different classes of ARVT. Whether such improvements translate to clinical benefit remains unclear. These findings suggest more work is required to rescue and restore the immune system in those infected by HIV-1, and immune therapy is a potential candidate, setting the stage for the studies described in Chapters Four and Five.

## CHAPTER FOUR: IL-2 THERAPY IN THE CONTEXT OF cART

### 4.1 The IL-2 Remune Study - Prospective, randomized, controlled, pilot study of the effect of Interelukin-2 therapy and therapeutic immunization on T-cell phenotype, activation and IL-2 receptor expression in conjunction with cART in HIV-1 infected individuals

*4.1.1 Introduction*

Interleukin-2 is a cytokine produced by activated T-cells which stimulates T-cells, NK cells and B cells. Exogenous administration has been shown to increase CD4 T-cell counts at all stages of HIV-1 infection (Kovacs *et al.,* 1996, Davey *et al.*, 2000, Losso *et al.,* 2000, Abrams *et al.,* 2002, Levy *et al*., 2003, Ruxrungtham *et al.*, 2004). Cyclical subcutaneous administration in HIV-1 infected individuals results in a significant and sustained increase in CD4 T-cell count, with a tolerable side effect profile in the majority of patients. It does this via a three chain cell surface receptor composed of the $\alpha$ chain (CD25), the $\beta$ chain (CD122) and the $\gamma$ chain (CD132). The IL-2 receptor exists in two forms, a high ($\alpha\beta\gamma$) and an intermediate ($\beta\gamma$) affinity receptor. These receptors are distributed differently on various cells. The resultant increase in T-cells is thought to be attributable to a combination of peripheral expansion, increased thymic output and decreased apoptosis, the relevant contribution of each of these factors is still a topic of considerable debate. Improvement in T-cell function has also been demonstrated with IL-2 therapy (*Levy et al*., 1999, 2003, Sullivan *et al*., 2003, Marchetti *et al*., 2004,), with improved proliferative capacity and a degree of anergy reversal. Concerns regarding the up-regulation of viral replication have been assuaged by a meta-analysis showing a reduction in viral load (Emery *et al*., 2000), probably attributable to the use of IL-2 in the context of cART, and lack of sustained elevation of plasma viraemia in the absence of cART (Youle et al., 2006).

Related concerns regarding hyperactivation also appear to be unfounded. (Kovacs et al., 1995, 1996, Carr et al., 1998, Hengge et al., 1998) Recent results have suggested this IL-2 expanded T-cell population may be qualitatively different to that resulting from antigenic-stimulated expansion (Sereti *et al*., 2000), being less anergic and more long lived. IL-2 receptors of high ($\alpha\beta\gamma$) and intermediate affinity ($\beta\gamma$) are up-regulated differentially on CD4 and CD8 T-cells by exogenous IL-2 administration. Cross sectional studies have suggested the IL-2 associated increase in CD25 expression is not sustained and the resulting CD4+CD25+ cells may be different to the immuno-regulatory CD4+CD25+ previously described (Tregs) (Sereti *et al*., 2002).

Recent studies have administered IL-2 therapy as an induction phase followed by a maintenance phase, the latter largely driven by the change in CD4 T-cell count. The median time to repeat the IL-2 cycle is typically 12 months (Kovacs *et al.*, 1996, Hengge *et al*., 1998, Miller *et al*., 2001, Abrams *et al*., 2002). There is little longitudinal data to offer more in-depth, detailed immunologically based support for this approach. This was the model used for the large international clinical endpoint trials of IL-2 therapy, ESPRIT and SILCAAT. These trials were designed to address whether the IL-2 induced increase in CD4 T-cell count translated into clinical benefit. Both were negative in this respect, however it may be that the strategy employed, the specific SIM utilized to drive maintenance therapy and the role of potential co-therapies need revisiting in order to fully assess the potential for IL-2 therapy.

Remune is a gp-120 depleted vaccine which has been investigated as a therapeutic vaccine in the setting of HIV-1 infection (Moss *et al.*, 1997, 1998, 2000, Churdboonchart *et a*l., 2000, Kahn *et al*., 2000, Sukeepaisarncharoen

*et al.*, 2001, Chantratita *et al*., 2004). The use of the vaccine in the context of this study was to safely introduce antigenic stimulation in the presence of a suppressed viral load and in the context of additional immunotherpay.

We therefore undertook a study to examine the immunological effects of IL-2 therapy and therapeutic immunization in the setting of fully suppressive cART. We aimed to describe the effect of exogenous IL-2 on IL-2 receptor and CD28 expression on T-cells, T-cell activation and naïve and memory T-cell populations. A sub-study was conducted and is detailed later in this chapter. Additional studies on LPR to mitogens, recall antigens and HIV-specific responses, TRECS and CCR5 receptors and β-chemokines were carried out in collaboration with colleagues and are published elsewhere (Burton *et al*., 2002, Pido-Lopez *et al.,* 2003, Hardy *et al*., 2003, 2007; listed in Chapter Eight).

*4.1.2 Patients and Methods*

4.1.2.1 Patients and cART

Patients were all adult HIV-1 infected, antiretroviral naive individuals attending a single out-patient department. The study was approved by the local research ethics committee and all patients gave written, informed consent. Patients commenced cART upon study entry with the choice of therapy decided by the trial physician (AKS) in consultation with the patient and their regular consultant. In each case cART followed current guidelines and consisted of at least two NA and at least one NNRTI or PI. Over time as a reflection of changing clinical practice patients were more likely to commence NNRTI based regimens, and those on PI therapy were switched to boosted PI regimens. Within the study, patients received routine and safety blood monitoring as per routine clinical care, in addition to study investigations.

4.1.2.2 IL-2 therapy and Remune immunisation

After 16 weeks of cART patients with a CD4 T-cell count ≥300 cells/μl and a viral load below the level of detection (VLBLD, <50 copies/ml) were randomized to one of four study arms, continue cART (Arm A), cART and IL-2 (Arm B), cART, IL-2 and Remune (Arm C) or cART and Remune (Arm D). Patients were also identified as belonging to Group 1 (IL-2 therapy, Arms B+C) or Group 2 (no IL-2 therapy, Arms A+D). IL-2 was administered at a dose of 5 MIU subcutaneously, twice daily for 5 days for three 4 weekly cycles. The IL-2 cycles were administered at weeks 17, 21 and 25. Prophylactic medication was offered and dose reduction employed to reduce side effects as required. Remune (100 μg) was administered by intra-muscular injection at weeks 17, 29, 41 and 53.

4.1.2.3 Delayed type hypersensitivity

Delayed type hypersenstivity (DTH) testing was performed at weeks -3, 17, 29 and 53. Skin testing using Remune antigen and control was performed to assess the clinical presence of HIV-1 specific cell-mediated immune responses.

4.1.2.4 Quality of Life (QOL)

Validated quality of life questionnaires of two types were administered at weeks 0, 16, 29 and 65 (Quality of Life, QoL) and weeks -3, 0, 12, 16, 21, 25, 41 and 65 (Impact of Symptoms Scale, ISS) respectively for all patients. Additionally patients receiving IL-2 also had ISS questionnaires on day 5 of each IL-2 cycle and week 29, and Remune recipients had ISS at week 53. The questionnaires were tailored to reflect the study arm. The questions were categorized into one of 5 groups for analysis: overall health, physical functioning, energy and fatigue, cognitive functioning and health distress.

Comparisons were made over time, between Arms A-D and between Groups 1 and 2.

4.1.2.5 HIV-1 plasma viral load and T-cell subsets and phenotype

Blood samples were taken pre-study (weeks -3 and -6) and at weeks 0, 4, 8, 16, 21, 25, 29, 41, 53 and 65 following commencement of cART. A substudy was performed in a number of patients receiving IL-2, when additional samples were obtained on the final day of each cycle of IL-2 therapy. Samples from each time point were analysed for lymphocyte subsets, phenotype and plasma HIV-1 RNA. Viral load was determined using the Quantiplex HIV RNA 3.0 branched DNA assay (Chiron Diagnostics, Halstead, UK). The assay has a detection limit of 50 copies/ml. The Epics XL-MCL (Beckman Coulter) was used for flow cytometric analyses of total $CD3^+$, $CD4^+$, $CD8^+$, $CD16^+/CD56^+$ (NK cells) and $CD19^+$ (B cells) lymphocytes, using whole blood. These were performed as routine laboratory investigations as described in Chapter Two.

Three colour flow cytometry was performed by AKS on CD4 and CD8 T-cells for naive (CD45RA+ on stored specimens, CD45RA+62L+ on fresh specimens) and memory (CD45RO+) T-cells, activation markers (HLA-DR+, CD38+), co-stimulatory molecule (CD28+), and the $\alpha$ and ß chains of the IL-2 receptor (CD25+ and CD122+ respectively). Receptor/molecule expression density was measured by mean fluorescent intensity (MFI). The three colour flow cytometry was performed on FACSCalibur (Becton Dickinson, UK) with Cell Quest software by gating on forward and side scatter plots and CD3+ cells. 10,000 gated events were collected, as described in section 2.9.

4.1.2.6 Statistics

FACS Data Analyses:

As longitudinal data on immunological markers were available, and since multiple assessments of these on the same subject were available at different study time points, within subject assessments of the immunological markers at different time points were expected to be correlated. This correlation needed to be accounted for when selecting analytical methods. The statistical analysis used needed to consider four main characteristics of the longitudinal data:

*1) Time may be an explanatory variable,*

*2) Repeat measurements for a subject are likely to be correlated,*

*3) The co-variables may be time-dependent (they may vary through time for a subject),*

*4) Missing data in the successive immunological markers measurements may induce a bias.*

A repeated measured linear mixed model was used to derive time adjusted slope or rate of change since randomisation to study time points of each immunological marker data collected by treatment arms. MIXED procedure in Statistical Analysis Software version 9.1.3 (SAS) was used by fitting values of the immunological markers from all study time points as a dependent variable. A random intercept model was fitted using MIXED procedure in SAS. The independent variables included the fixed effects of subjects and study visit time points. This assumes that the intercepts for each patient are random and the effects of changes (slopes) in immunological markers from baseline, or entry into the cohort, are also random and differ between patients. A compound symmetry covariance matrix was used to model the within subject errors. This assumes that the variances are homogeneous.

There is a correlation between two separate measurements, but it is assumed that the correlation is constant regardless of how far apart the immunological marker measurements were over time. Estimates of immunological marker slopes were obtained from subjects by time interaction and each immunological marker has been presented pictorially, over time and by study arms, as point estimates. The trends over time have been presented firstly by 4 treatment arms and secondly grouped by 2 arms. All statistical analyses were carried out in SAS version 9.1 and all significance tests are two tailed.

QOL analysis:

QOL questions were grouped into QOL items. Questions in each item were recoded so that the highest category was represented as worse for that item while a low value in the category was represented as converse. All missing responses to items were scored 0. Due to a varying number of questions appearing in each QOL items, these have been standardised. The total scores from each QOL item responses were taken as a fraction of the observed QOL item responses relative to the expected total QOL item responses score. These have been expressed as that out of 100. So the minimum possible score that could be observed in each grouped QOL item could be 0 and the maximum possible score that could be observed is 100. These scores standardised to 100, which means that if a QOL item score was "100" this implied that there was a 100% 'worse' outcome for that QOL item. The tests were all non parametric due to the small sample size. Between group comparisons were tested using Kruskal-Wallis test, while within patient comparisons for changes in QOL between time points were tested using the Wilcoxon test.

*4.1.3 Results*

A total of 65 patients initially consented to the study and were screened. Sixteen withdrew consent between weeks -6 and 0, electing to defer commencing cART, and were excluded. Of the 49 who commenced week 0, 7 patients subsequently withdrew from the study prior to randomization at week 16, two for treatment of concurrent conditions (lymphoma and hepatitis), one due to psychiatric illness, one for side effects of cART and three for personal reasons, including satisfaction with CD4 T-cell count response to cART alone. Six patients failed to achieve a CD4 T-cell count of 300 cells/μL prior to randomisation and were withdrawn from the study. Results for all these patients were included in the week 0–16 analysis. Thirty-six patients were randomized, with the disposition Arm A 10, Arm B 8, Arm C 9 and Arm D 9, there were no statistically significant differences in their baseline characteristics (Table 4.1). Fifteen patients (Group 1) received 46 cycles of IL-2. Two patients declined IL-2 therapy after randomisation due to satisfaction with T-cell count response to cART alone. One patient was lost to follow up after week 29 due to transfer to another centre and declined to permit follow-up through that centre.

**Table 4.1: Baseline characteristics of IL-2 Remune study patients who were randomized**

| | **All** | **GROUP 1 n = 17** | | **GROUP 2 n = 19** | |
|---|---|---|---|---|---|
| Arm | 36 | **B** | **C** | **A** | **D** |
| Mean age (years) | 36.09 | 36.25 | 37.00 | 38.71 | 34.89 |
| Sex (Male/Female) | 35M 1F | 7M 1F | 9M | 10M | 9M |
| Median CD4 T-cell count (cells/μl) | 303 | 345 | 303 | 337 | 239 |
| Median viral load (copies/ml) | 84,603 | 99,295 | 84,603 | 92,795 | 39,917 |
| No. on PI based cART | 16 | 4 | 4 | 3 | 5 |
| No. on NNRTI based cART | 20 | 4 | 5 | 7 | 4 |

Results of this study are presented for the whole group up until week 16. Week 16-65 data are presented for Groups 1 and 2 and for Arms A-D. Given the small numbers, wide variation in data points and some loss to follow up and non-adherence to cART, many observed differences cannot be shown to be significant, having resulting wide confidence intervals. As a pilot study, therefore, differences that are expected, consistent or plausible are highlighted as potentially worthwhile of future investigation.

4.1.3.1 CD4 T-cell count, percentage, naïve and memory CD4 T- cells

After commencing cART, as expected, there was an increase in the total number and percentage of CD4 T-cells, including naïve cells over the first 16 weeks of therapy (Figure 4.1A). CD4 T-cell count rose by 189 cells over the 16 weeks (95% CI 119.4 - 259.1) and the percentage rose by 6.6 (95%CI 4.1 – 9.1). The percentage of naïve CD4 T-cells rose by 1.84% (95%CI -0.01-3.7). Despite an initial early rise, at 16 weeks no change was seen in the proportion of CD4 effector T-cells. Patients in Group 1 who received IL-2 demonstrated further increases in their CD4 T-cell count compared to those in Group 2 who did not receive IL-2 (Figure 4.1B).

**Figure 4.1A: Change in CD4 T-cell count for all patients completing week 16 in the IL-2 Remune study, n=46**

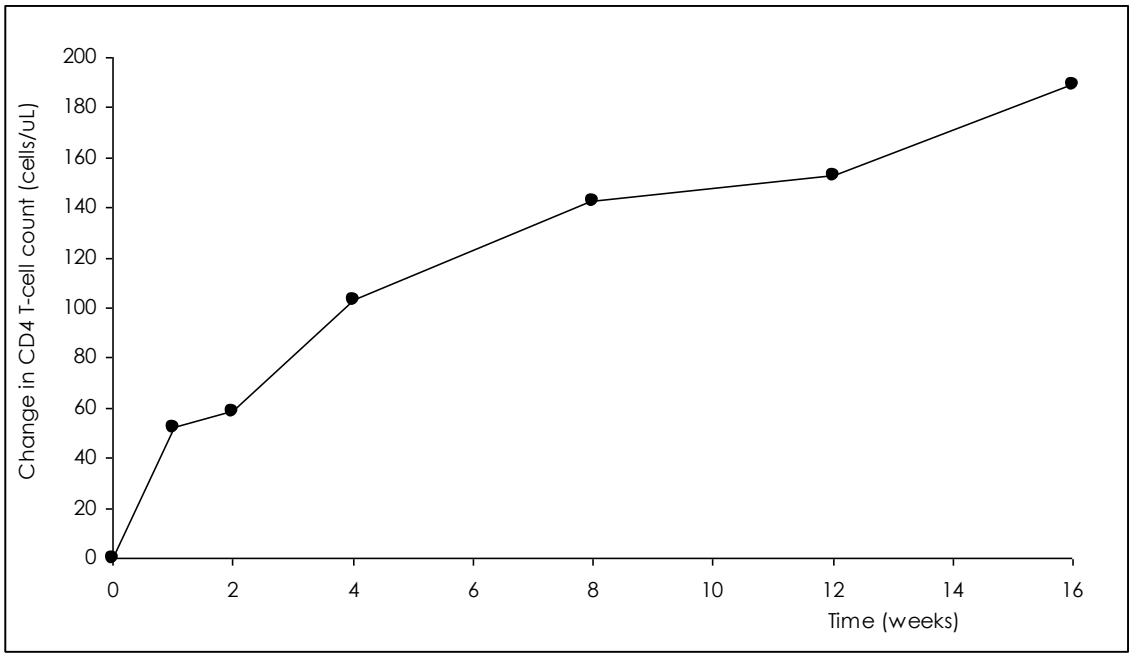
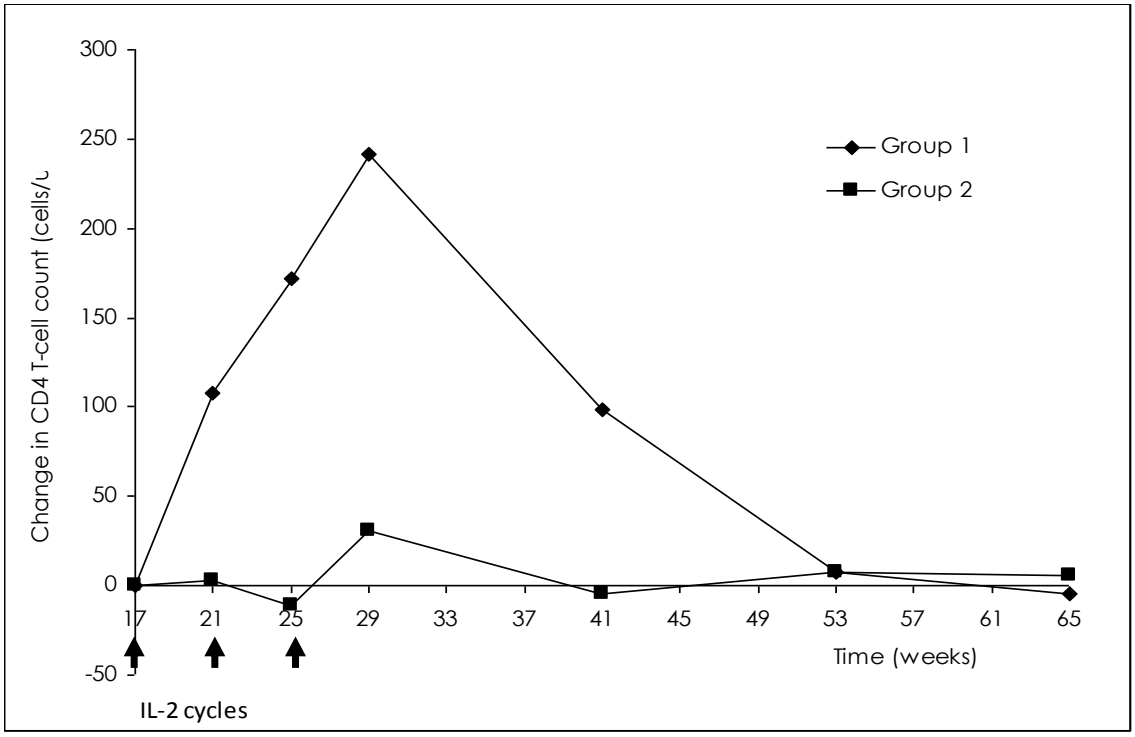



**Figure 4.1B: Change in CD4 T-cell count for patients in Groups 1 (IL-2 therapy, n=17) and 2 (no IL-2 therapy, n=19) from week 17 to 65 of the study**



The peak effect was seen at week 29, four weeks after the third and final IL-2 cycle (+241.7 cells [95%CI 112.1 – 371.2]). Although not as great, the difference was still observed at week 41, 16 weeks after the last cycle of IL-2, but had disappeared by week 53. This was similar for the CD4%, except that the difference between the two groups, although reduced was still observed at week 65. The increase was seen in both CD45RA+ and CD45RO+ T-cells, with the increase being of a greater magnitude in the latter subset. The increase in both these subsets was greatest immediately following the cycles of IL-2. When looking at the 4 study arms, the difference in the CD4 T-cell count and % appeared to be driven mainly by Arm C (IL-2 and Remune) and was sustained. For the effector subset both Arms B and C had peri-IL-2 cycle increases, however, despite the initial rise being greater in Arm B it was only sustained in Arm C. Details of the changes seen in CD4 T-cells and percentage and naïve and effector CD4 T-cells for Groups 1 and 2 for weeks 16-65 are shown in Table 4.2, including 95% confidence intervals, as an example of the source of the results described below.

**Table 4.2: Change from baseline CD4 T-cell count and %, naïve and memory/effector T-cells week 16-65**

| Study Group | CD4 T-cell count (cells/µL) | SE | Lower 95% CI | Upper 95% CI | CD4 T-cell % | SE | Lower 95% CI | Upper 95% CI | CD4+CD45RA+ | SE | Lower 95% CI | Upper 95% CI | CD4+45RO+ | SE | Lower 95% CI | Upper 95% CI |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| GROUP 1 | | | | | | | | | | | | | | | | |
| Week 16 | | | | | | | | | | | | | | | | |
| 21 | 107.98 | 66.08 | -21.54 | 237.50 | 4.24 | 2.27 | -0.20 | 8.68 | 5.54 | 1.86 | 1.89 | 9.18 | 15.56 | 3.10 | 9.49 | 21.63 |
| 25 | 172.27 | 64.74 | 45.38 | 299.16 | 8.16 | 2.22 | 3.81 | 12.51 | 4.43 | 1.86 | 0.79 | 8.08 | 8.48 | 3.10 | 2.41 | 14.55 |
| 29 | 241.67 | 66.10 | 112.11 | 371.23 | 8.21 | 2.27 | 3.77 | 12.65 | 5.88 | 1.82 | 2.32 | 9.45 | 7.47 | 3.03 | 1.53 | 13.42 |
| 41 | 98.28 | 66.10 | -31.29 | 227.84 | 6.63 | 2.27 | 2.19 | 11.08 | 1.92 | 1.86 | -1.73 | 5.57 | 3.16 | 3.10 | -2.92 | 9.23 |
| 53 | 8.00 | 66.10 | 121.57 | 137.56 | 2.24 | 2.27 | -2.20 | 6.69 | 1.62 | 1.86 | -2.02 | 5.27 | 4.07 | 3.10 | -2.00 | 10.14 |
| 65 | -4.48 | 67.62 | 137.01 | 128.06 | 1.58 | 2.32 | -2.96 | 6.13 | 4.71 | 1.96 | 0.86 | 8.56 | 8.12 | 3.26 | 1.74 | 14.51 |
| GROUP 2 | | | | | | | | | | | | | | | | |
| Week 16 | | | | | | | | | | | | | | | | |
| 21 | 2.80 | 65.10 | 124.79 | 130.38 | 0.30 | 2.22 | -4.65 | 4.06 | -0.31 | 1.81 | -3.85 | 3.24 | 2.10 | 3.08 | -3.94 | 8.14 |
| 25 | -10.55 | 66.43 | 140.76 | 119.65 | 0.35 | 2.27 | -4.09 | 4.79 | -1.54 | 1.81 | -5.08 | 2.01 | -0.18 | 3.08 | -6.22 | 5.87 |
| 29 | 31.13 | 63.88 | -94.09 | 156.34 | 1.44 | 2.18 | -2.83 | 5.71 | 0.51 | 1.77 | -2.95 | 3.98 | 0.31 | 3.02 | -5.62 | 6.24 |
| 41 | -4.44 | 63.88 | 129.65 | 120.78 | 2.43 | 2.18 | -1.84 | 6.70 | 0.99 | 1.85 | -2.64 | 4.62 | 1.07 | 3.15 | -5.10 | 7.23 |
| 53 | 7.19 | 63.88 | 118.03 | 132.40 | 0.88 | 2.22 | -3.47 | 5.23 | 0.86 | 1.73 | -2.54 | 4.26 | -0.30 | 2.97 | -6.13 | 5.53 |
| 65 | 5.53 | 65.08 | 122.02 | 133.08 | 0.21 | 2.22 | -4.56 | 4.14 | 1.12 | 1.81 | -2.42 | 4.67 | 0.61 | 3.08 | -5.43 | 6.65 |

CI = Confidence Interval
SE = Standard Error

4.1.3.2 CD8 T-cells, percentage, naïve and memory cells

During weeks 0 - 16, the total number and percentage of CD8 T-cells decreased as expected; the fall in percentage was statistically significant, however the fall in total cell count was not (-5.3% [95%CI -9.7 - -0.9] and -15 cells [95%CI -227 - +197] respectively). A decrease was observed in CD8+CD45RO effector T-cells by week 8 and further by week 16 (-8.3% [95%CI -13.7 - -2.9]). Subsequent IL- 2 therapy resulted in further reductions in Group 1 which were maintained to week 65. Looking at the 4 study arms, both IL-2 therapy arms experienced reductions in total counts and %, which were sustained in Arm C but not B. The differences observed associated with each IL-2 cycle appeared to be more in the effector subset (CD45RO) than naïve (CD45RA) CD8 T-cells.

4.1.3.3 Activated CD8 T-cells

Over the first 16 weeks of cART the percentage of activated CD8 T-cells decreased, with decreases observed in both HLA-DR and CD38 expression. The reduction in CD8+HLA-DR+ T-cells was significant by week 8 and further reduced by week 16 (-5.3% [95%CI -8.4 - -2.2]) (Figure 4.2A). HLA-DR receptor density had also declined by week 8 (-8.2 [95%CI 12.9 - -3.6]) although this was returning towards baseline by week 16. CD8+CD38+ reduction reached significance by week 4 and further reduced out to week 16 (-16.2% [95%CI -22.4 - -9.9]. Similarly to HLA-DR the CD38 receptor density declined significantly at week 8 (-55.7 [95%CI -110.4 - -1.1]) but returned towards baseline by week 16. Activation markers increased with each IL-2 cycle as expected. These elevations returned to baseline, and for Group 1 the CD8+ HLA-DR+ T-cell count was below baseline by week 65 (Figure 4.2B), in both Arms B and C (Figure 4.2C).

**Figure 4.2A: Change in CD8+HLA-DR+ T-cell count for all patients completing week 16 in the IL-2 Remune study**

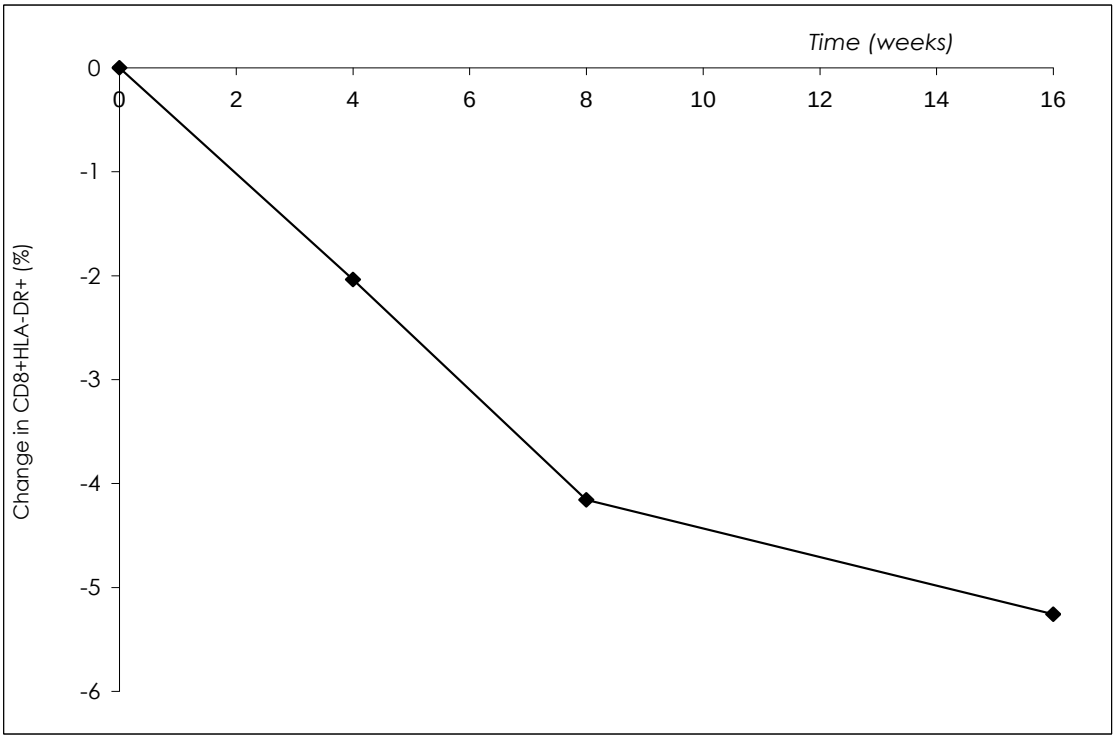



**Figure 4.2B: Change in CD8+HLA-DR T-cell count for patients in Groups 1 (IL-2 therapy) and 2 (no IL-2 therapy) from week 17 to 65 of the study**

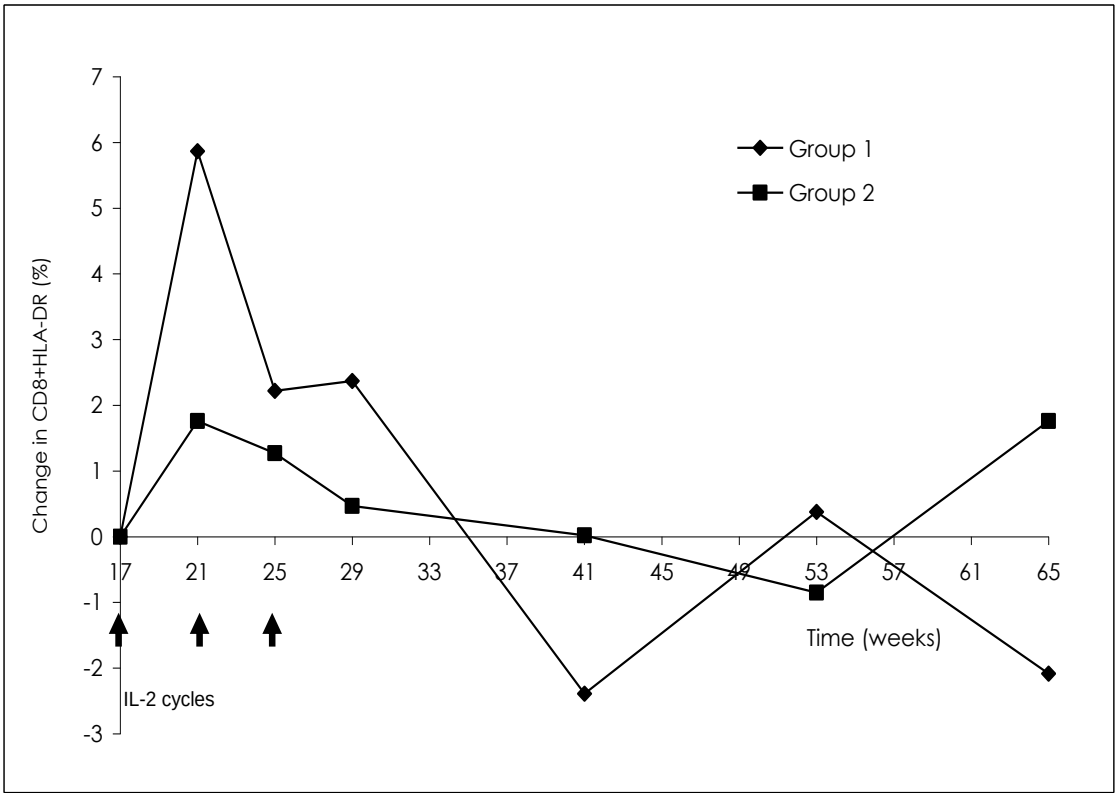
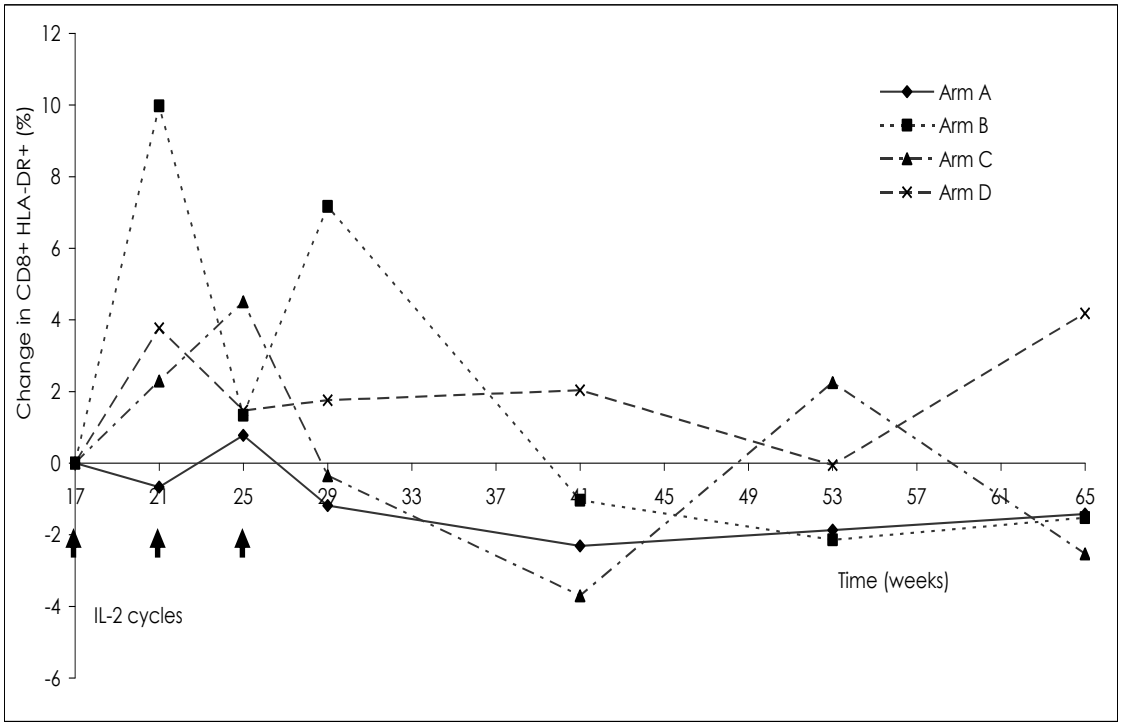


**Figure 4.2C: Change in CD8+HLA-DR+ T-cell count for patients in Arms A-D from week 16 to 65 of the study, n= A=10, B=8, C=9, D=9**




There were 6 patients whose CD4 T-cell count failed to recover above 300 cells/μL and were excluded prior to randomisation. The mean CD8+CD38+ % for these 6 patients was 49.0 *vs* 38.9 at week 0 *vs* week 16, with a mean change of -9.1. The patients who were randomised within the study had mean % of 36.0 *vs* 17.8 respectively and a mean difference of -18.3.

4.1.3.4 Co-receptor expression

cART did not appear to have any effect on CD4+CD28+ expression in the first 16 weeks of the study. A small rise was seen in CD8+CD28+ T-cells by week four (1.8% [95%CI -0.8 – 4.4]) and although maintained it did not reach significance. There was an increase in receptor density expression by week 8 of 5.2 (95%CI 1.8 – 8.6). The percentage of CD4+CD28+ T-cells increased in Group 1 associated with IL-2 cycles, and the difference persisted to week 65. In Arm C density of receptor expression rose with each cycle and was increased at week 65.

The proportion of CD8+CD28+ cells rose over the first 16 weeks, as did the receptor density on these cells. Arm C again had an increase following each IL-2 cycle and was still elevated at week 65. Arm B also saw an increase, although this was neither significant nor consistent. Receptor density expression was increased for both arms at week 65.

4.1.3.5 IL-2 receptor expression

In the first 16 weeks of cART a small increase was seen in CD4+CD122+ T-cells, although this was not significant (+.67% [95%CI -.69 - +2.01]). There was no change in CD122 expression on CD8 T-cells. An initial rise at week four in CD4+CD25+ T-cells (1.24 [95%CI 0.1-2.4]) was not sustained out to week 16, and there was no change in CD8+CD25+ T-cells.

Examining IL-2 receptor expression after week 16, the percentage of CD4+CD122+ lymphocytes rose immediately following IL-2 administration in Group 1 but returned towards baseline by week 29. By week 65 however they had again increased above baseline and were greater compared to Group 2. A similar rise was seen with each cycle for CD8+CD122+, returning to baseline by week 41. Focusing on the CD25 receptor, the proportion of CD4+CD25+ T-cells increased with IL-2 therapy (Group 1 and Arms B and C) compared to Group 2 (and Arms A and D) (Figures 4.3A and B). Although this decreased slightly at week 41, there was a further rise out to week 65. The receptor density on CD4 T-cells mirrored these changes (Figures 4.3C and D).

**Figures 4.3A and B: Changes in CD4+CD25+ from week 16 to 65 of the study for patients in A) Groups 1 and 2 and B) Arms A-D**

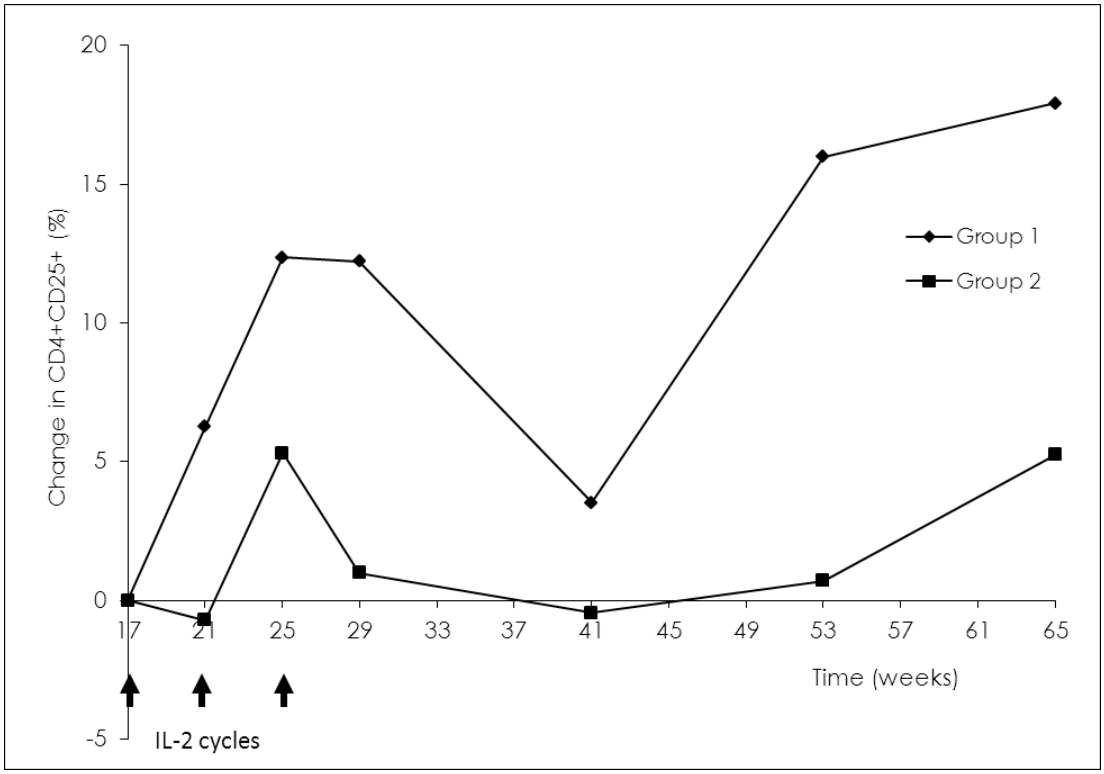
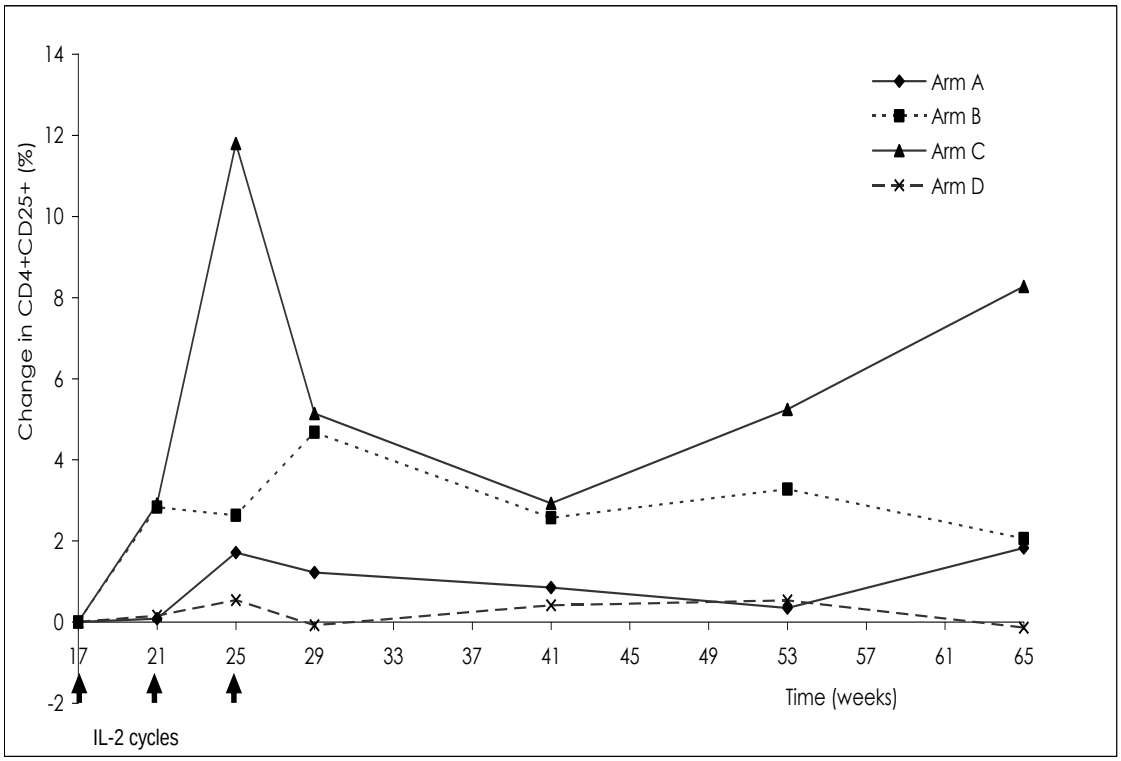



**Figure 4.3B**




**Figures 4.3C and D: Changes in CD4+CD25+ MFI from week 16 to 65 of the study for patients in C) Groups 1 and 2 and D) Arms A-D**

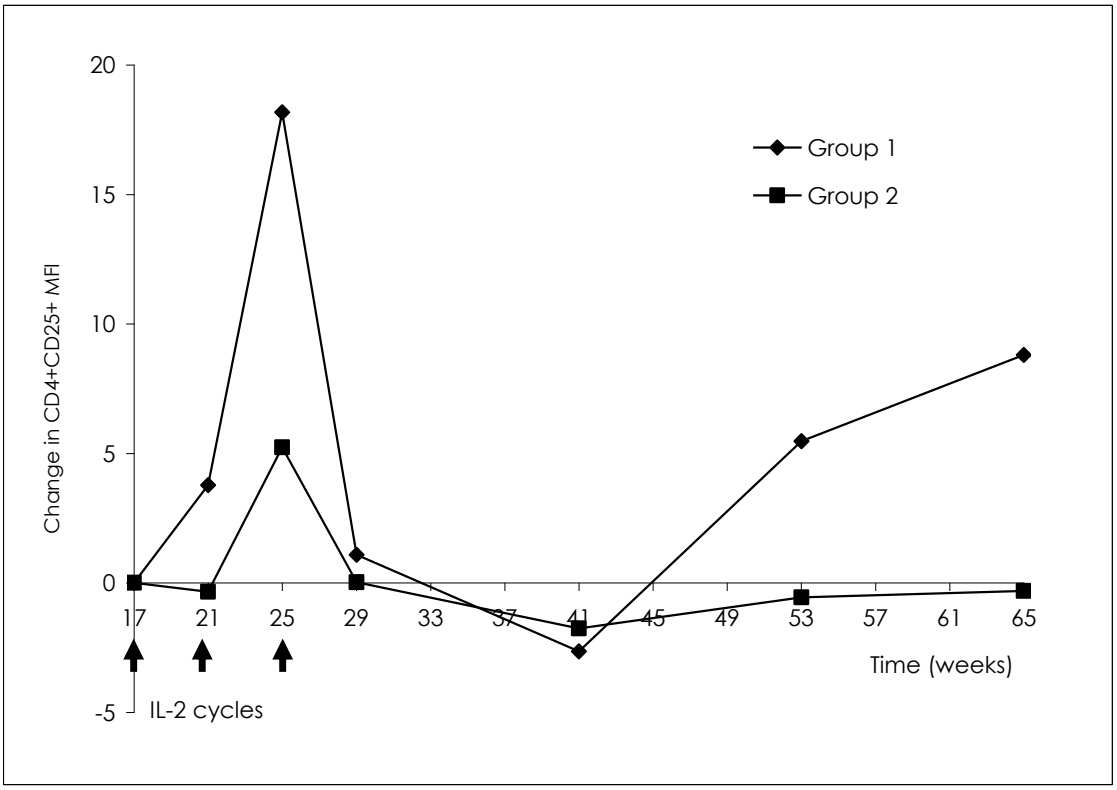
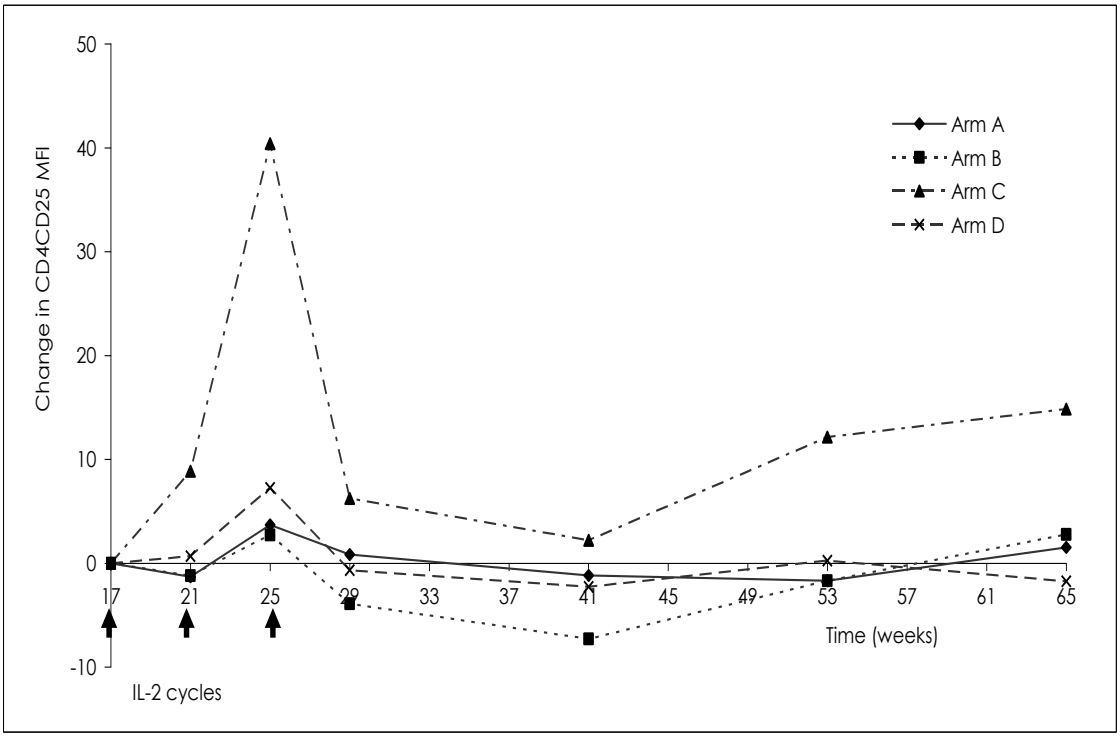



**Figure 4.3D**




4.1.3.6 B cells and NK cells

B cells (CD19+) rose following the commencement of cART by 73 cells (95% CI 38.5-107.4) over 16 weeks; following randomization, there was no further change, and no difference observed between the two groups.

Commencing cART did not effect NK cells, however following IL-2 administration a significant increase in NK cells was observed, reaching a peak for Group A at week 29; 55.2 cells (95% CI 15.7-94.6), with no significant change observed in Group B.

4.1.3.7 Viral load

At baseline the median viral load was 84,603 copies/ml, at week 16 it was below the level of detection (BLD) and by week 65 the median was BLD with 85% of patients having an undetectable VL. There were no differences between the groups or arms. Viral load did increase in those patients who received IL-2 at the time of the IL-2 cycle but this increase was not sustained (detailed further in the substudy which follows). Of the five patients with a detectable virus at week 65, three had a level under 300 copies/ml plasma.

4.1.3.8 DTH

One patient in Arm C demonstrated a significant reaction following skin testing with Remune at week 29, with an average diameter of 10mm of induration and 15.5 mm of erythema, but this did not recur at week 53. Two further patients had induration diameters of 7.5mm; both at week 29 and both in Arm C. Another 9 patients experienced some measurable reaction, either induration or erythema or both but to lesser degrees, 1 in Arm C, 4 in Arm A, and 2 in each of Arms B and D.

4.1.3.9 Side effects and adverse events

Two patients withdrew after randomisation due to concurrent conditions requiring treatment, hepatitis and lymphoma. Of patients receiving IL-2 the majority experienced well recognised side effects which were controlled

with prophylactic medications in the majority of cases. One patient had IL-2 dose reduction after an episode of hepatitis likely due to IL-2 therapy, one declined the third cycle due to depression and one patient, previously diagnosed with asthma, experienced an acute attack during one cycle. One patient in Arm A, experienced lactic acidosis likely to be secondary to stavudine therapy and had a treatment interruption, and one patient in Arm D experienced viral breakthrough and changed his ARV regimen due to underlying drug resistance.

4.1.3.10 Quality of Life Questionnaires

There was a high baseline level of functioning and quality of life with low levels of health distress. This was maintained with no significant changes over the first 16 weeks of cART. Following randomisation there were no significant changes observed over time, or between the arms or groups at each time point and over the study period. In patients who received IL-2 there was an increase in scores (worsening) at the end of each cycle, particularly for energy and fatigue (*e.g.* cycle 2= +20 [15-25]; median [IQR]) and health distress (*e.g.* cycle 3= +20 [18-23]) in Arm C, but these changes were not significant. Qualitative data revealed that although many patients found the IL-2 therapy difficult in terms of side effects they expressed very positive effects in terms of mental attitude and perceived health benefits; *e.g.* 'IL-2 has been very beneficial although the side effects were a nightmare', 'The main change is to self-esteem and outlook as a result of the CD4 count improving beyond all expectation'

*4.1.4 Discussion*

This longitudinal, randomized, controlled pilot study confirms previously reported effects of exogenous IL-2 (Buter *et al.,* 1993, Kovacs *et al.,* 1996, Davey *et al*., 1997, Carr *et al*., 1998, Hengge *et al*., 1998, Arno *et al*., 1999,

Sereti *et al*., 2000), but also demonstrates several interesting potential additional effects of IL-2 and possibly of therapeutic immunisation. As a pilot study there was an insufficient number of patients in each arm to determine the significance of many of the observed differences, however this study does have several findings that warrant further investigation. In some instances the changes observed in the two Groups appear to be driven more by Arm C (IL-2 and Remune) than Arm B (IL-2 alone), which is also observed in relation to the sustainability of changes. Again, due to the small number of patients in each group, direct conclusions cannot be made but it raises possible areas of interest for future larger studies.

Following the introduction of cART the CD4 T-cell count rose, as expected. As previously described this is likely to represent redistribution, with the early rise in effector cells (Arno *et al*., 1999), increased production, with the rise in naïve cells (Arno *et al*., 1999, Levy *et al*., 1999), and prolonged survival of both due to decreased cell death with the reduction in immune activation (Kovacs *et al.*, 1996, Carr *et al*., 1998). IL-2 further augmented this rise. The pattern and difference between the Arms is interesting. The IL-2 associated rise is closely linked to the cycles of IL-2 and is not sustained past 53 weeks, although the rise in CD4 T-cell % is sustained to week 65. This suggests if using the CD4 T-cell count as the SIM driving further cycles of therapy that the treatment interval required is likely to be less than the 12 months currently being suggested. Although CD4 T-cell may be the SIM being used currently, the results of ESPRIT and SILCAAT (Abrams *et al*., 2009) suggest this may not be the correct driver of IL-2 re-administration. Despite the small numbers and wide confidence intervals, it is of interest that the IL-2+Remune Arm (Arm C) appeared to be driving this increase, and the increase was seen to be sustained in Arm C. Speculating as to the reason behind this raises the

possibility that the 'benign' antigenic stimulation of Remune (administered at weeks 17, 29, 41 and 53) confers an additional drive to increase CD4 T-cells; also observed in the Thai studies (Churdboonchart *et al*., 2000, Sukeepaisarncharoen *et al*., 2001), especially as the increase was observed to a greater degree in the effector cells. It is interesting to speculate whether the two immune therapy interventions interacted synergistically; either by IL-2 expanding Remune primed T-cells, or by increasing naïve cells susceptible to the antigenic stimulation subsequently afforded by the vaccination.

The initiation of cART led to a decrease in CD8 T-cells, which was augmented by IL-2 therapy, sustained in Arm C and observed in effector cells more than naïve. Levels of activation decreased with the introduction of cART; there was also a reduction in the receptor density expression observed. The level of CD8 T-cell activation rose in association with the IL-2 cycles. It may be this is a direct effect of IL-2 or it may be a result of the peri-cycle IL-2 viral load blips we observed (as described in the next section of this chapter [Sullivan *et al*., 2003]). Interestingly Group 1 had levels of activation below baseline by week 65, which suggests a potential additional benefit of exogenous IL-2, and may be of interest as an alternative SIM to monitor IL-2 therapy. This may be of increasing relevance with the increasing importance of so-named non-HIV related morbidity and mortality; the associated conditions being in some way linked to immune system activation (El-Sadr *et al*., 2008, Kuller *et al*., 2008, Baker *et al*., 2010, Nauhaus *et al*., 2010). The small number of patients who did not achieve a CD4 T-cell count of 300 cells/μL and were therefore not randomized, had higher CD8+CD38+ T-cell counts at week 16, and had experienced a decline on therapy only half that seen in the randomized group. This level of activation may explain the observed blunted immune recovery and has been reported in patients experiencing a discordant CD4

response as described in Chapter Three (Garcia *et al*., 2000, Leng *et al*., 2001, Anthony *et al*., 2003). It is interesting to speculate whether IL-2, by decreasing activation over time, may enhance CD4 T-cell recovery in addition to the mechanisms already described.

IL-2 therapy resulted in an increase in the proportion of cells expressing the co-receptor CD28, again more marked in Arm C. Given the role of the CD28 receptor in T-cells activation signaling (*Wang et al.,* 2004, Corthay, 2006), this would suggest IL-2 can influence qualitative as well as quantitative immune recovery.

There was a sustained increase in the high affinity IL-2 receptor expression on CD4 T-cells following each IL-2 cycle and over the study period. The proportion of CD4+CD25+ T-cells also increased with each cycle and overall. This phenotype was the contemporary definition for Tregs, however they are now most commonly defined by the phenotype CD4+CD25+CD127$^{lo}$FoxP3+ (Seddiki *et al*., 2006, Liu *et al*., 2006, Girdlestone *et al*., 2007). The original Treg characterisation by the phenotype CD4+CD25+ proved problematic as CD25 is also expressed on non-regulatory T-cells during immune activation. It was subsequently recognised that these cells co-expressed Forkhead family transcription factor FoxP3. The addition of absence or low cellular surface expression of CD127 refines the definition of this T-cell sub-population. There is ongoing debate regarding this definition, with several further refinements suggested by various authors. This is because Tregs thus defined are phenotypically and functionally heterogeneous, with only a minority subset being potent suppressor cells (Chen *et al*., 20011). Other proposed cell surface markers include CTLA-4 (cytotoxic T-lymphocyte associated molecule-4) and GITR (glucocorticoid-induced TNF receptor), however their functional significance is unknown. Currently the search continues to better

characterise this functionally distinct population of T-cells by identifying markers that are uniquely and specifically expressed on FoxP3-expressing regulatory T cells; to date this has been largely unsuccessful. Some recent work based on DNA methylation analysis holds promise as these elusive cells have a demethylated region within the FoxP3 gene which is unique to Tregs (Wieczorek *et al*., 2009). Tregs have a role to play in modulating the level of immune activation. However work by others (Sereti *et al*., 2000) suggests the cells produced in response to exogenous IL-2 therapy in the absence of antigen are behaviorally different to the classically described T regs. While this may be so, and other defining investigations for Tregs were not carried out within this study, it is difficult to further comment on the potential complex interplay between the various cell types involved. Concurrent with the observed increase in Tregs associated with each IL-2 cycle there was also an increase in activation, viral load and HIV-1-specific responses (as described in the next section of this chapter). These observations would be consistent with Tregs in this context being less immuno-suppressive but clearly is not evidence of a direct relationship as it is likely there are many factors at play. Over the study period however the rise in Tregs was maintained, the HIV-1 responsiveness sustained and activation had decreased. Thus whilst there is no direct evidence as to the immunosuppressive effect, or lack thereof, of this population, it may be postulated that the antigenic stimulation provided by either the Remune vaccination or the peri-IL-2-cycle low level vireamia influenced these cells in some way. It is also possible that the correlation between the increase in the CD4+CD25+ subset (and transient increase in CD4+CD122+) and the increase in the anti-HIV-1 responses suggest that IL-2 may be able to break the anergy of HIV-1-specific T-cells. This however is speculative given the small patient numbers and wide data ranges and it is

not possible to determine the role of the addition of Remune and whether this was a causative factor in the differences observed.

This increase in receptor expression and its duration would appear to lend support to the approach of induction/maintenance IL-2 therapy albeit at different intervals, but an alternative driver for repeat cycles may need to be found.

**4.2 Sub-study - Interleukin-2 associated viral breakthroughs induce HIV-1-specific CD4 T-cell responses in patients on cART. (Reproduced here as published in AIDS (Sullivan *et al.*, 2003, see Chapter 8, with additional data).**

Intermittent subcutaneous low dose interleukin-2 (IL-2) in combination with cART has been shown to increase CD4 T-cell counts at all stages of disease (Arno *et* al., 1999, Davey *et al.*, 2000). It appears however that eradication of virus is unlikely unless additional immune strategies can be employed to both target latently infected cells and enable qualitative HIV-1 specific immune reconstitution (Markowitz *et al.*, 1999). Animal models suggest provision of antigenic stimulation will be essential to this endeavour (Lori *et al.*, 2000). Investigation of structured treatment interruptions and therapeutic immunisations currently offer little promise and the former has all but been abandoned due to the inherent risks of uncontrolled viral rebound, CD4 T-cell loss, reseeding of viral reservoirs and emergence of drug resistance (Pai *et al*., 2005, Eron, 2008). To date little success has been achieved in chronic infection with therapeutic immunization alone (Kahn *et al*., 2000, Autran *et al*., 2008, Wijesundara *et al*., 2010).

Fourteen of the patients in the IL-2 Remune study were included in this sub-study; we additionally aimed to assess the acute effect of IL-2 on plasma VL and HIV-1-specific responses. Patients gave written informed consent and were commenced on cART 16 weeks prior to IL-2 therapy, when a VBLD (50 copies/ml) was required. IL-2 was administered at a dose of 5 MIU subcutaneously, twice daily for 5 days, for three 4-weekly cycles. Samples were obtained on days 1 and 5 of each IL-2 cycle for CD4 T-cell count and VL, and at weeks 0, 4, 8, 12 and 24 for CD4 T-cell count, VL and LPR to HIV-1 recombinant antigens (*nef*, gp120 and p24) (Imami *et al.*, 1999). Results are expressed as a stimulation index (SI) where a positive result is a SI $\geq$5. In

addition to the data published in AIDS I also performed flow cytometry as described in the main study in this chapter.

Fourteen patients received a total of 42 cycles of IL-2. Nine of the 14 patients had at least one day 5 viral load blip with a total of 13 blips (range 51–355 copies/ml). Mean viral load rose for all IL-2 cycles, day 1 *vs* day 5: 50 *vs* 91 copies/ml (p=0.002) (Figure 4.4). Mean CD4 T-cell count rose from 430 to 1073 cells/μL (p=0.001). Mean SI for HIV-1-specific antigens rose above 5 at week 24 (Figure 4.5). The proportion of patients with a positive SI to any HIV-1 antigen rose from 31% to 54% by week 24.

**Figure 4.4: Plasma HIV-1 RNA load for individual patients, ■ = mean, ▨ = IL-2 therapy**

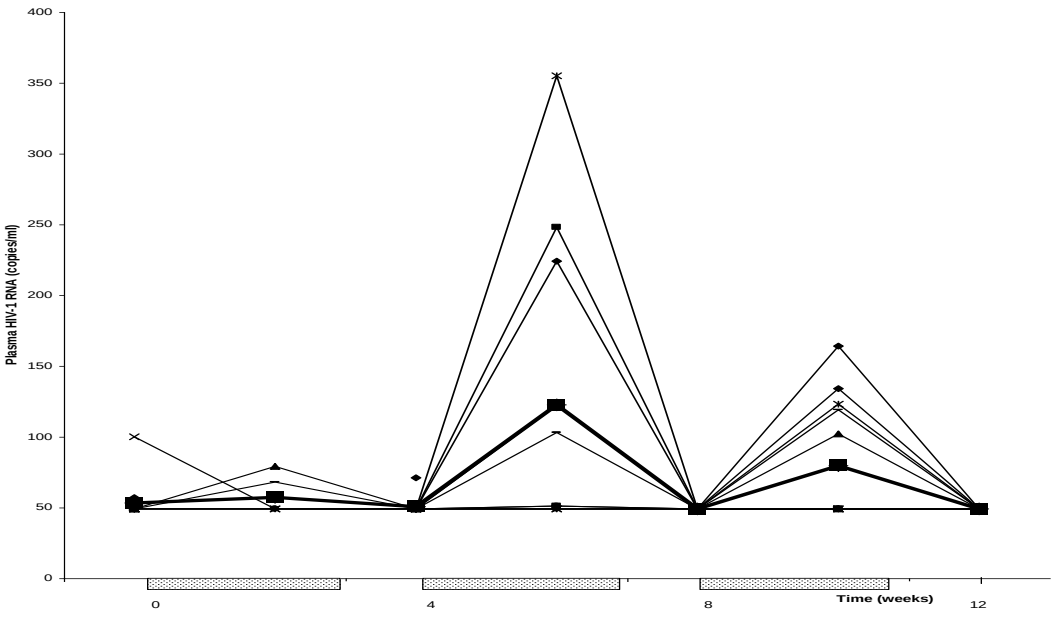
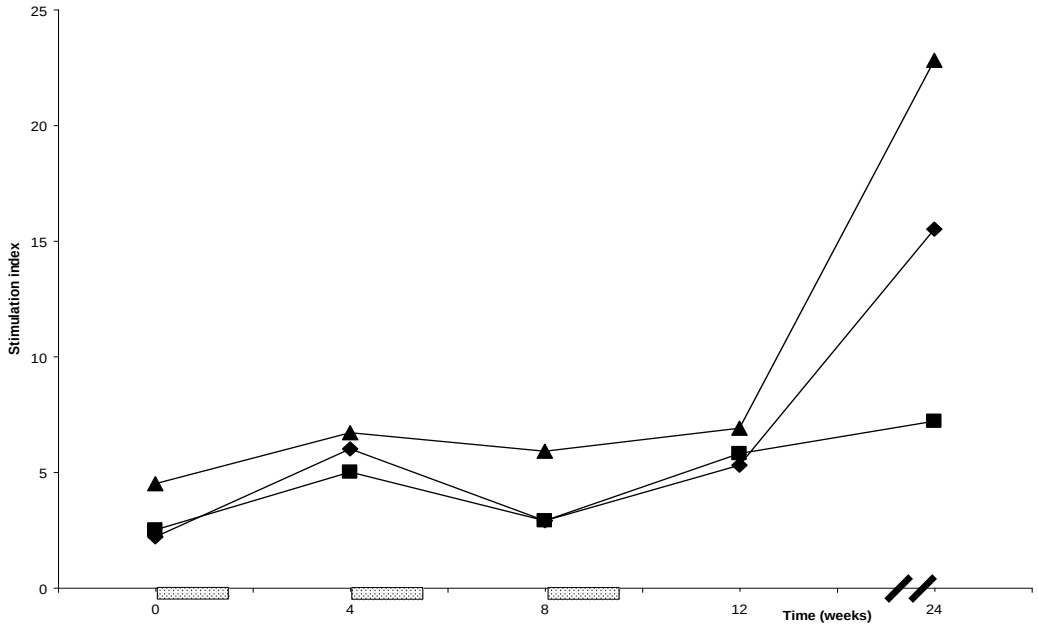
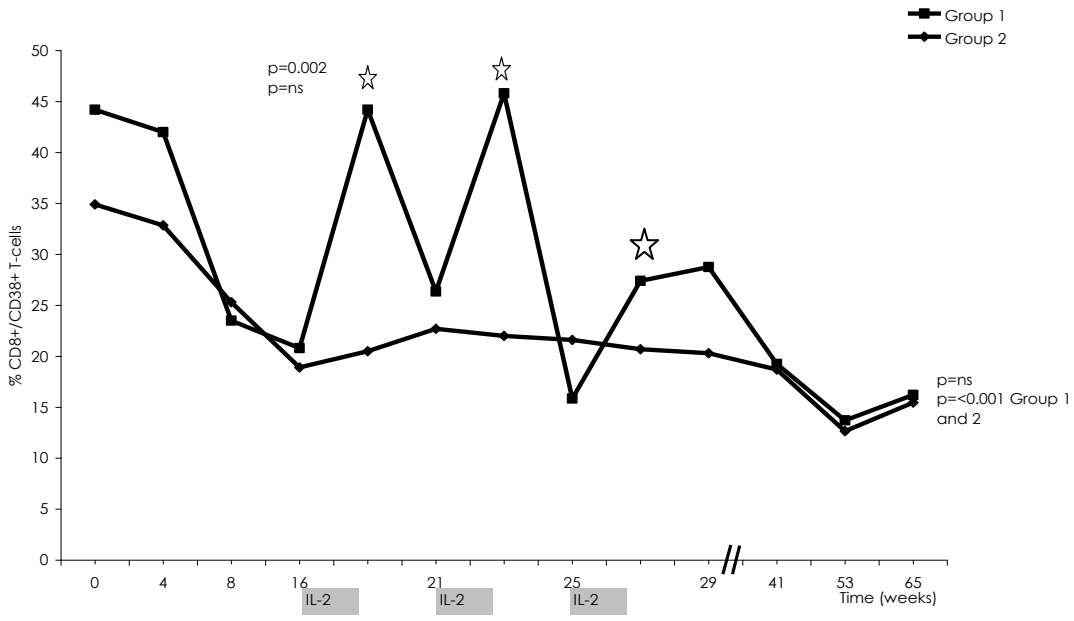


**Figure 4.5: Mean HIV-1-specific responses, ◆=*nef*, ■ =gp120, ▲ =p24, ▭ =IL-2 therapy**



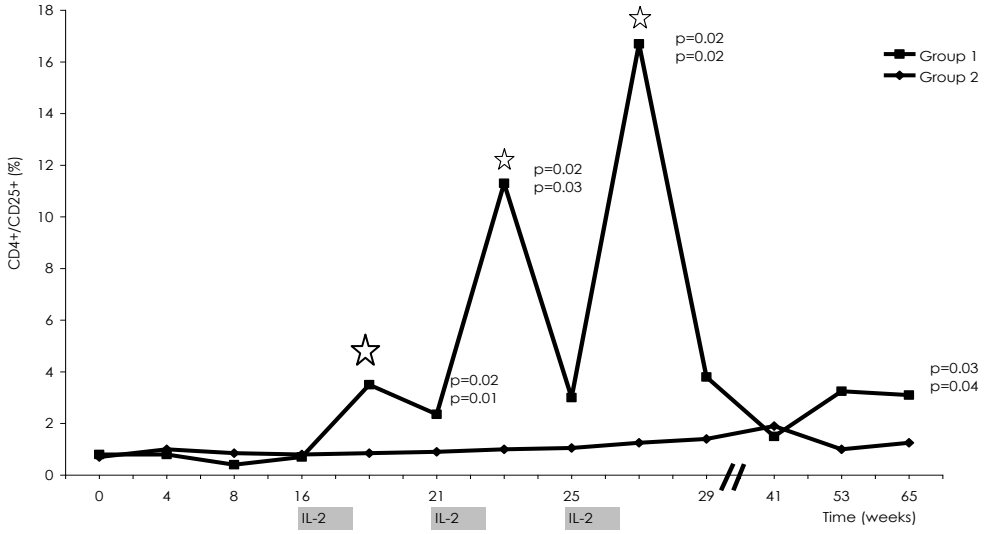

Figures 4.6 and 4.7 show data additional to that contained in the AIDS letter; demonstrating the IL-2 cycle-related rise in CD8+CD38+ and CD4+CD25+ respectively.

**Figure 4.6: Median CD8+CD38+ T-cell (%) for patients in Groups 1 and 2**




IL-2 therapy ▭ , upper p = Group 1 *vs* Group 2, lower p = Group 1 *vs* baseline, ☆ day 5 of IL-2 cycle

**Figure 4.7: Median CD4+CD25+ T-cell (%) for patients in Groups 1 and 2**

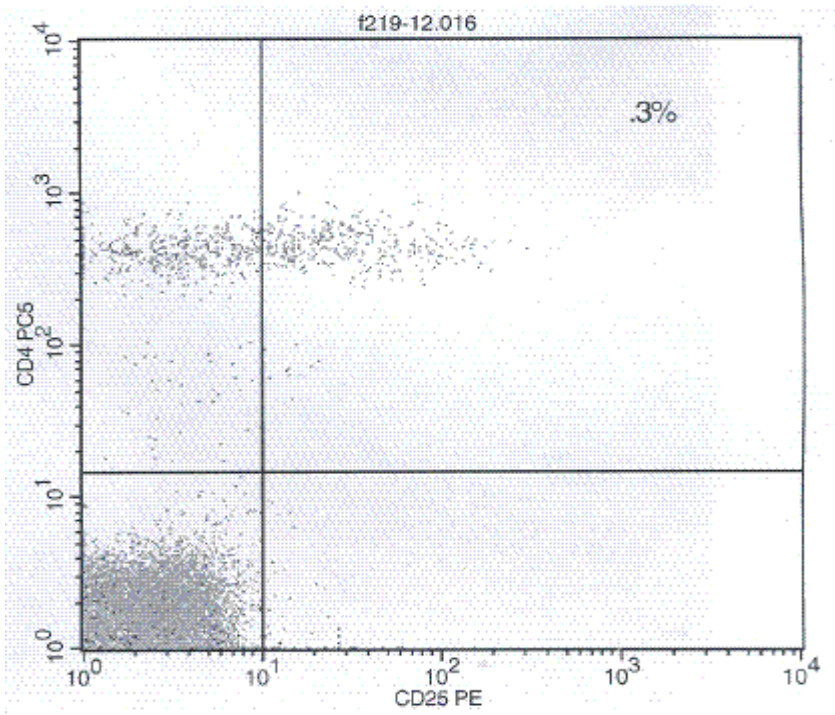



IL-2 therapy , upper p = Group 1 *vs* baseline, lower p = Group 1 *vs* Group 2, ☆ day 5 of IL-2 cycle

Figures 4.8 and 4.9 are reproductions of the flow cytometry results for IL-2 receptors on days 1 and 5 for a single patient, albeit representative. In Figure 4.8B three distinct populations can be seen, representing (left to right) resting, activated and regulatory CD4 T-cells (Girdlestone *et al*., 2007).

**Figure 4.8: Flow cytometric representation of CD4+CD25+ T-cell subpopulations on A) day 1 and B) day 5 of IL-2 cycle for a single representative patient**

**4.8A**




**4.8B**

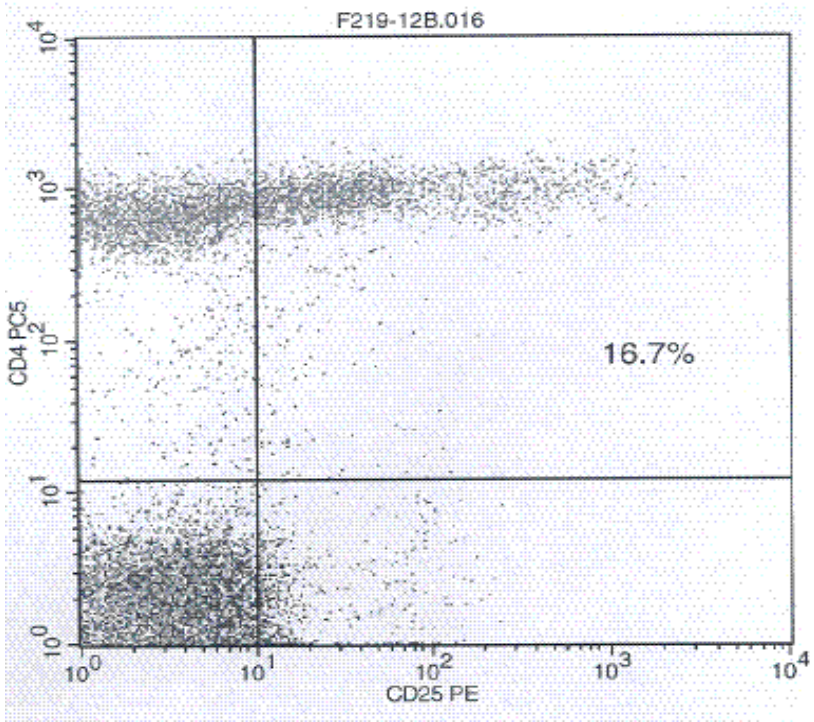
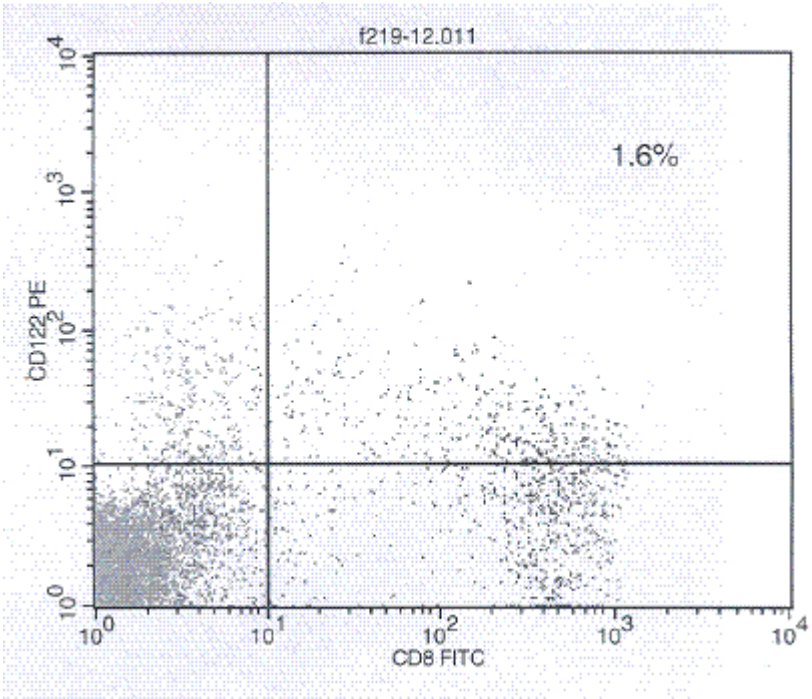
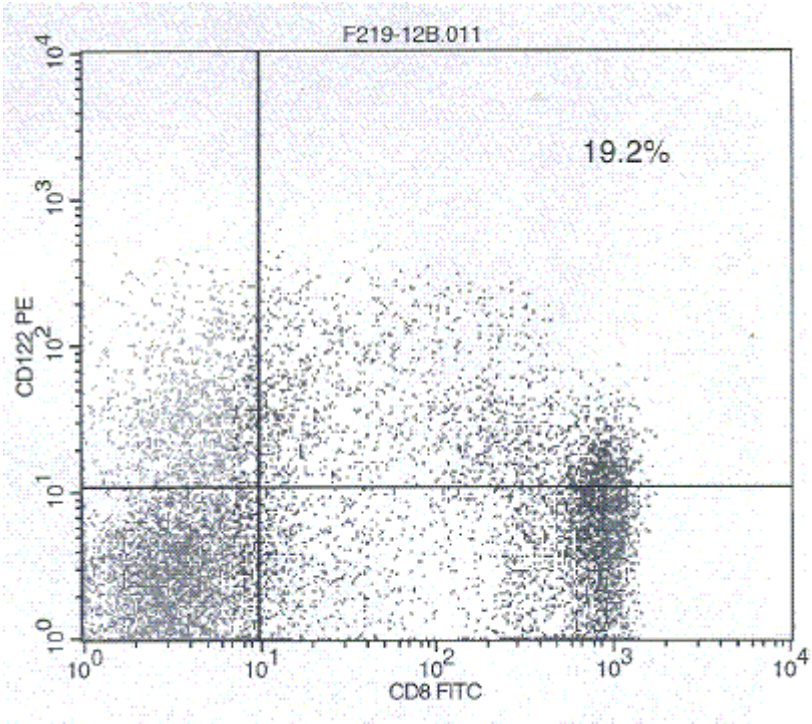


**Figure 4.9: Flow cytometric representation of CD8+CD122+T-cells on A) day 1and B) day 5 of IL-2 cycle for a single representative patient**

**4.9A**




**4.9B**



*4.2.1 Discussion*

For the first time we have shown low dose intermittent IL-2 induces viral breakthroughs in the context of fully suppressive cART in chronic infection. These blips are controlled and are closely followed by an increase in HIV -1-specific responses, which are maintained out to 24 weeks. This suggests a possible role for IL-2 as a safe method of autoimmunisation in contrast to treatment interruption (Eron, 2008); providing autologous virus in the context of cART while preventing significant viral rebound, protecting CD4 T-cells, preventing reseeding of reservoirs and purging latently infected cells. Several major questions remain unanswered; the detailed dynamics and possible minimum threshold required of IL-2 associated viral blips, the duration of the induced HIV-1-specific responses, the potentially harmful effect of even this low level viraemia on immune system activation and potentially the reseeding of viral reservoirs, and most importantly causation needs to be established between these two sequential, reproducible phenomena. Answers should enable the creation of immunotherapy treatment schedules which augment the advances seen in HIV care since the introduction of cART.

**4.3 Summary**

This Chapter has examined the effects at a cellular level of combined immunotherapy in the context of fully suppressive cART. The IL-2 Remune study reports novel findings of the acute effects of IL-2, including induction of HIV-1 specific responses. Overall increases were observed in CD4+, CD4+CD28+ and CD4+CD25+ T-cells, with some reduction in activation levels. Tregs are of particular interest as the precise function of these cells in HIV-1 infection and following IL-2 therapy is still to be fully defined. A trend

was observed for several effects to be more marked and more sustained in the arm combing all three treatments; suggesting a therapeutic potential for combination immune therapy which is worth pursuing. Recent work investigating the potential role for other cytokines in HIV-1 infection, such as IL-7, showed potentially beneficial effects on both CD4 and CD8 T-cells, predominantly memory, but no effect on Tregs (Sereti *et al*., 2009). This further supports the premise that, similar to ARVT, it will be through combination immunotherapy that we may achieve more robust immune restoration and begin to observe a clinical benefit.

# CHAPTER FIVE: IL-2 IN THE ABSENCE OF FULLY SUPPRESSIVE cART – DOES IT HAVE A ROLE?

## 5.1 Introduction

Before the advent of effective cART the use of IL-2 in HIV-1 infected individuals yielded relatively poor results in clinical trials. The majority of these studies were in patients on non-suppressive regimens and such patients were also likely to have high levels of ARV drug resistance and immune activation. Subsequent trials in the presence of suppressive cART have shown significant improvement in several SIM, however this did not appear to translate into clinical benefit in two large trials (Abrams *et al*., 2009), although one of these studies was in patients with low nadir CD4 T-cell counts, and the other had a minimum CD4 T-cell count requirement of only 300 cells/μL, which would now be considered advanced disease. This chapter examines two potential scenarios where IL-2 may have a role to play in the absence of fully suppressive cART. The first is in patients with 'early' HIV-1 infection to explore the possibility of delaying the initiation of treatment by maintaining an individual's CD4 T-cell count. The second scenario is in patients with advanced disease, heavily pre-treated with multiple antiretroviral combinations and with few or no remaining treatment options, to see whether IL-2 can provide a buffer period while waiting for the availability of new therapies to enable construction of a potentially effective ARV regimen.

## 5.2 The Vanguard Study

Vanguard was a multi-centre, randomised, controlled pilot study of subcutaneous intermittent interleukin-2 without concurrent antiretroviral therapy in subjects infected with HIV-1; Chelsea and Westminster Hospital

was one site, recruiting 6 patients, 5 of whom received IL-2 (see Statement of Co-authorship Paper 3, page 3). The full published paper follows Chapter 8.

*5.2.1 Introduction*

The use of cART clearly affords significant improvement in both morbidity and mortality as detailed in the Introduction (Chapter One). This success however is limited by side effects, long term toxicity (the full effects of which are still emerging), viral resistance, and the fact that once initiated therapy is life long; with adverse outcomes associated with treatment cessation or interruption. Thus there is a need for the development of alternate therapies or treatment strategies. There is data which suggests that as a single parameter, the CD4 T-cell count may be a more important predictor of clinical outcome than plasma viral load (Mocroft *et al.*, 2000), and this is supported by the data presented in Chapter Three of this thesis. In line with these data is the investigational approach of administering intermittent IL-2 to augment or preserve immune function (Stein *et al.*, 1993, Lederman *et al.*, 1995, Emery *et al.*, 1996). Despite some concerns regarding rises in plasma HIV RNA levels when IL-2 is administered, there is sufficient evidence to demonstrate that when this occurs it is a transient phenomenon, as demonstrated in the IL-2 Remune sub-study in Chapter Four, and also reported by others (Kovacs *et al.*, 1996, Davey *et al.*, 1997,1999, 2000, Carr *et al.*, 1998, Levy *et al.*, 1999, Losso *et al.*, 2000, Ruxrungtham *et al.*, 2000, Abrams *et al.*, 2000). In fact, a meta-analysis has demonstrated in patients on cART IL-2 produced larger decreases in viral load than antiretroviral therapy alone (Emery *et al.*, 2000). One randomised study similarly found that IL-2 in combination with antiretroviral therapy produced larger decreases in VL than ARV therapy alone (Davey *et al.*, 2000) although these findings were

not observed in other randomised studies of short duration (Kovacs *et al.*, 1996, Davey *et al.*, 1997, 1999, Carr *et al.*, 1998, Levy *et al.*, 1999, Losso *et al.*, 2000, Ruxrungtham *et al.*, 2000, Abrams *et al.*, 2000). We observed no difference in plasma viral load in patients on ARVT between those who received IL-2 and those who did not (IL-2 Remune study, Chapter Four).

Vanguard was a multicentre, randomised, open-label, dose ranging trial of IL-2 in HIV-1 infected individuals in whom cART was not clinically indicated. The purpose of this randomised controlled pilot trial was to determine if intermittent IL-2 therapy administered without concomitant antiretroviral therapy increased CD4 T-cell counts. If this strategy were to be successful it might lead to a delay in the time at which chronic antiretroviral therapy would need to be initiated.

This chapter presents the results from the entire cohort to provide the context (for full study details refer to Chapter 8), as well as additional data from patients enrolled at the Chelsea and Westminster Hospital. The purpose of this nested study was to perform additional T-cell phenotypic analysis, similar to that done in the IL-2 Remune study (Chapter Four), so as to observe the effect of IL-2 on patients on and off cART, and specifically to observe the effect of IL-2 on T-cell activation in the absence of cART. This was to address theoretical concerns regarding exogenous IL-2 causing further immune stimulation of an already hyperactivated immune system. Again this focussed on CD38; which on CD8 T-cells indicates activation of the immune system, often due to viral replication, correlates with plasma viral load and is a recognised prognostic factor (Liu *et al*., 1997). On CD4 T-cells it indicates activation and proliferation, and may also be a marker of susceptibility to HIV-1 infection. We also examined the co-receptor CD28, a co-stimulatory molecule with a role in apoptosis (Li *et al*., 2010); those cells which are CD28

negative are thought to be anergic (Thomas *et al*., 2005, Rudd *et al*., 2009). CD25 was also measured as the balance between activated T-cells and Tregs (CD4+CD25+) may play an important role in controlling the immune response in a setting of permanent activation (Kinter *et al*., 2004).

*5.2.2 Patients and Methods*

Participants were recruited as part of the multi-centre UK study; the Vanguard study. Adult HIV-1 infected individuals who were ARV naïve with a minimum CD4 T-cell count of 350 cells/μL were eligible if they had never received IL-2 therapy and had no previous AIDS defining diagnosis or recent immunosuppressive therapy. The study had local Research Ethics Committee approval.

Patients were randomly assigned in equal proportions across the recruiting centres to intermittent subcutaneous injections of IL-2 at two different doses, 4.5 MIU or 7.5 MIU twice daily for five days every eight weeks, or no treatment. Given the side effect profile of IL-2 it was not possible to have a placebo arm. Side effects were managed with prophylactic medication and dose modifications in decrements of 1.5MIU.

Over the 24-week study period, all subjects were evaluated monthly with an additional visit on day 5 of each cycle for patients receiving IL-2 therapy. All patients were monitored clinically and routine safety bloods and T-cell subsets and viral load measurements were performed. Absolute CD4 and CD8 T-cell counts and plasma VL were performed at a single, central study laboratory (Urdea *et al.*, 1993, Dewar *et al.*, 1994, Connelly *et al.*, 1995, Mercolino *et al.*, 1995). The VL assay was a branch-chain DNA assay (Chiron) with a lower limit of detection of 50 copies/ml.

In the nested local sub-study additional phenotypic analysis was performed as described in Chapter Two. In brief the CD4 and CD8 compartments were

analysed for percentages and MFI of naïve and memory T-cells (CD45RA and RO), activation markers (HLA-DR and CD38), co-receptor CD28 and IL-2 receptors (CD122 and 25).

Treatment failure was prospectively defined as detailed in the full paper (Chapter 8).

### 5.2.3 Data analyses

The details of the statistical analysis for the entire cohort are described in the Vanguard paper (Chapter 8), and analysis is on an intention to treat basis.

The primary end points of the study were area under the curve (AUC) for CD4 T-cell count change from baseline and AUC for plasma HIV-1 RNA change from baseline over the 24 weeks of the study. Secondary end points included treatment failure, changes in percentage of CD4 T-cells, the number and percentage of CD8 T-cells, the CD4/CD8 T-cell ratio and safety data.

### 5.2.4 Patient disposition

A total of 45 subjects were screened; 9 were ineligible (one prior AIDS diagnosis and 8 low CD4 T-cell counts) and 36 were enrolled. Twelve were randomly assigned to each of the interleukin-2 treatment groups (giving a total of 24 IL-2 recipients) and 12 to the control group. The baseline characteristics of the three groups were similar (Table 5.1) and of the 36 enrolled patients, 35 were male with a mean age of 34.5 years. Baseline mean CD4 T-cell count for the entire cohort was 407 cells/μl blood (range 361-692) and plasma viral load was 35,827 copies/ml (range 6,641-236,518). Locally two patients were excluded (one with a prior *Mycobacterium tuberculosis* diagnosis, and one with a low CD4 T-cell count) and 6 patients

were enrolled; one to 7.5 MIU dose, four to 4.5 MIU dose and one to the control arm.

**Table 5.1: Demographic and baseline characteristics***

| | Treatment Group n = total cohort (***local cohort***) | | | |
|---|---|---|---|---|
| | Control | 4.5MIU IL-2 | 7.5MIU IL-2 | Combined IL-2 |
| N | 12 ***(1)*** | 12 ***(4)*** | 12 ***(1)*** | 24 ***(5)*** |
| Mean age (years) | 38.0 | 31.8 | 36.9 | 34.4 |
| Mean weight (kgs) | 71.4 | 71.7 | 74.7 | 73.2 |
| Number female | 0 ***(0)*** | 0 ***(0)*** | 1 ***(0)*** | 1 ***(0)*** |
| % homosexual | 100 ***(100)*** | 91.7 ***(100)*** | 83.3 ***(100)*** | 87.5 ***(100)*** |
| % antiretroviral naïve | 100 ***(100)*** | 100 ***(100)*** | 100 ***(100)*** | 100 ***(100)*** |
| Median CD4 T-cell count (cells/μL) | 477 | 423 | 388 | 393 |
| Median nadir CD4 T-cell count (cells/μL) | 447 | 396 | 352 | 372 |
| Median plasma HIV RNA (log copies/ml) | 4.1 | 4.2 | 4.4 | 4.3 |

***** *adapted from Vanguard paper (Chapter 8)*

5.2.5 Exposure to IL-2

Twenty four patients received 61 cycles of IL-2, two declined any IL-2 post randomization, three patients received only one cycle (two lost to follow-up [LTFU] and one citing personal reasons) and one patient received only two cycles. The cohort's IL-2 exposure is detailed in the Vanguard paper (Chapter 8). Locally, one patient received only 2 cycles (7.5 MIU and 4.5 MIU, average unit dose 4 MIU) and the remaining 4 patients completed three cycles each, with one dose reducing due to side effects; the average dose was 4.2 MIU (Table 5.2).

**Table 5.2: Exposure of subjects, shown by number of subjects commencing each cycle and average unit dose in each cycle, to IL-2 by treatment group.***

Treatment group; n = total cohort (***local***)

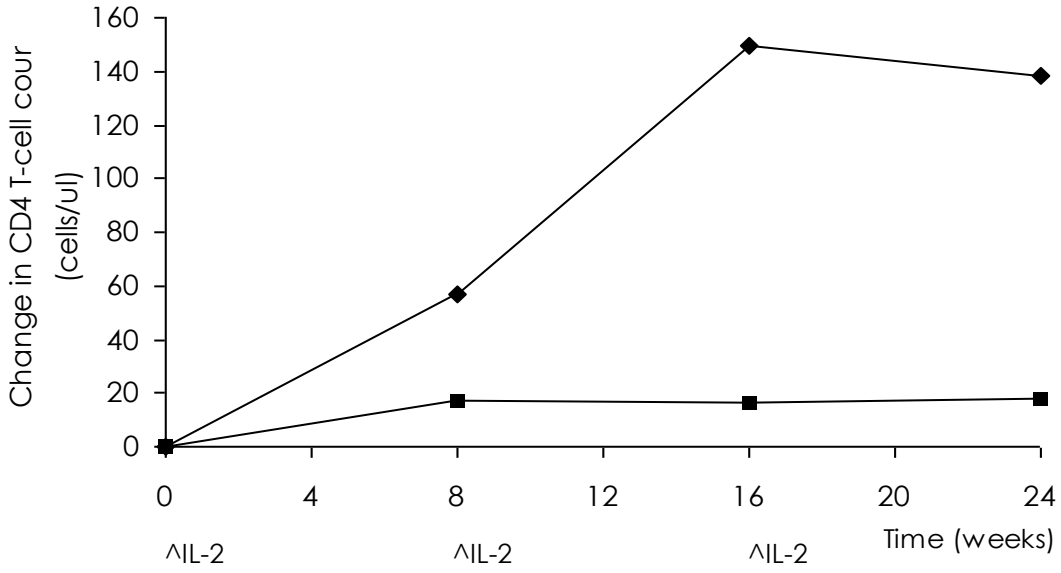
| | 4.5MIU | | 7.5MIU | |
|---|---|---|---|---|
| Cycle number | Number starting cycle | Average unit dose (MIU) | Number starting cycle | Average unit dose (MIU) |
| 1 | 11 ***(4)*** | 4.5 | 11 ***(1)*** | 7.3 ***(7.5)*** |
| 2 | 9 ***(4)*** | 4.4 | 11 ***(1)*** | 6.6 ***(4.5)*** |
| 3 | 9 ***(4)*** | 4.2 | 10 ***(0)*** | 5.8 ***(0)*** |

***** *adapted from Vanguard paper (Chapter 8)*

5.2.6 CD4 T-cell count

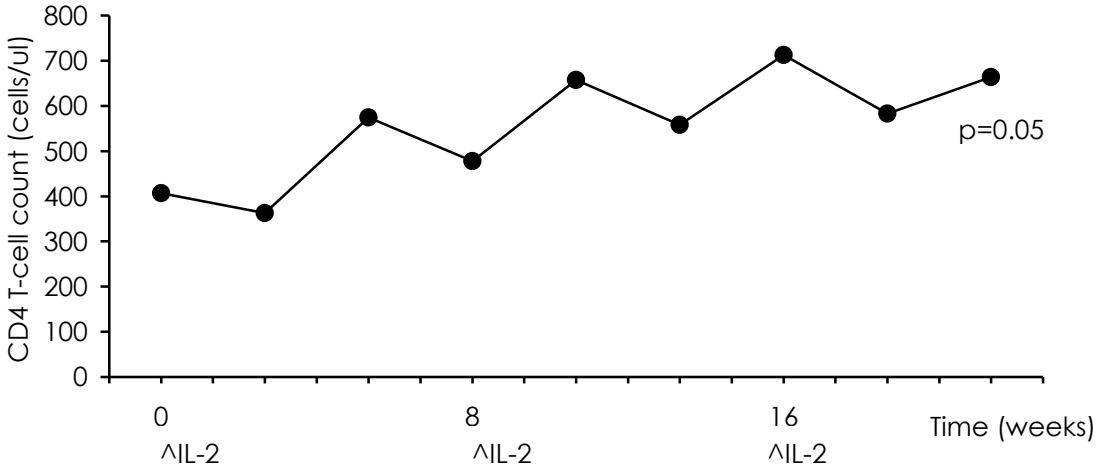
CD4 T-cell counts increased significantly in the IL-2 treatment groups, both in number and AUC. Changes from baseline and median CD4 T-cell counts for patients receiving IL-2 are shown in Figures 5.1 and 5.2. The numbers of CD8 T-cells in each treatment group remained stable throughout the study period in each group.

**Figure 5.1: Change in CD4 T-cell count from baseline**




♦ = IL-2 treatment group, ■ = control, ^ IL-2 indicates IL-2 therapy administered

**Figure 5.2: Median CD4 T-cell count in patients receiving IL-2 therapy**

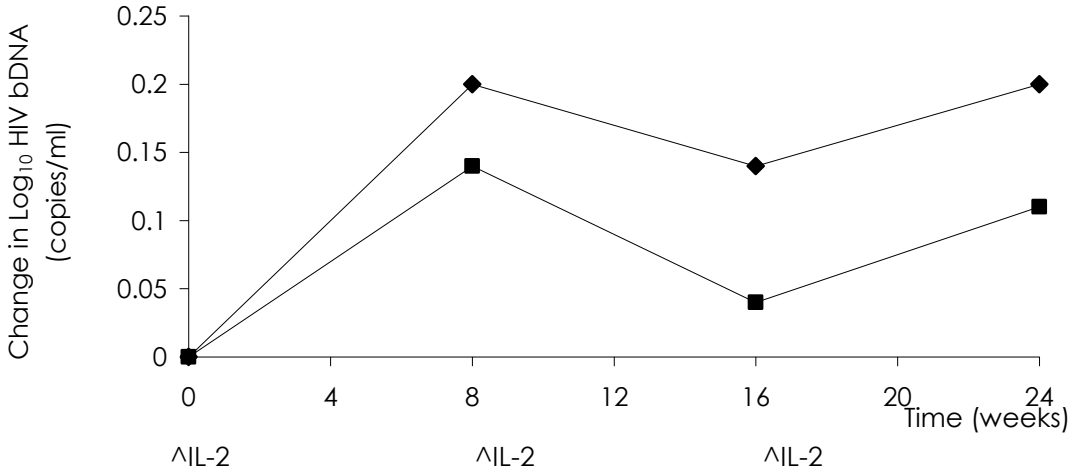



^IL-2 indicates IL-2 therapy administered

5.2.7 Plasma Viral Load

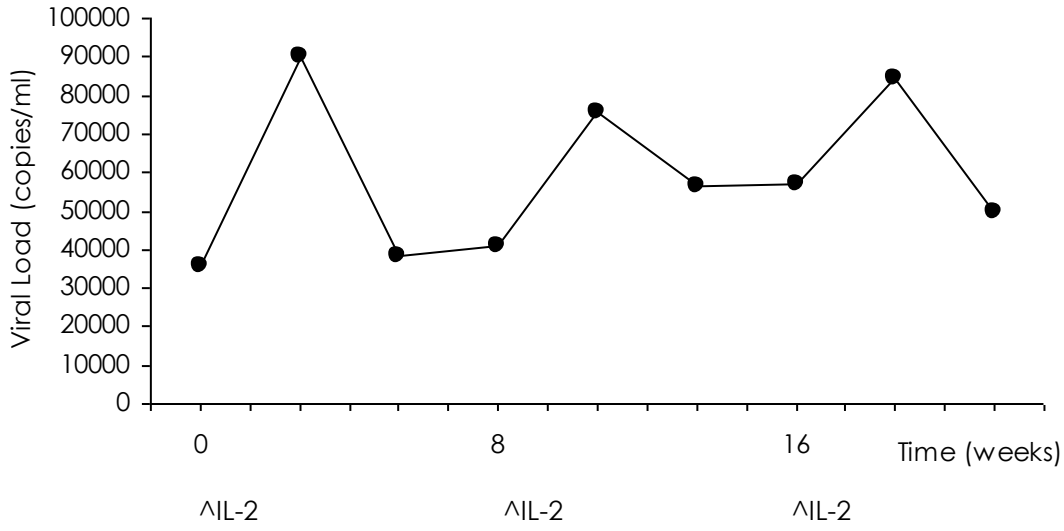
Viral load AUC in the whole cohort did not differ from baseline for both IL-2 treated patients and the controls, nor was there any difference between groups for mean changes at any time point. Figures 5.3 and 5.4 show change in VL from baseline and median VL throughout the study in those patients receiving IL-2. Day five analysis showed transient elevations of VL in 32-40% of IL-2 treated patients, with no differences observed between the two different IL-2 dose groups.

**Figure 5.3: Change in viral load from baseline**




♦ = IL-2 treatment group, ■ = control

**Figure 5.4: Median viral load in patients receiving IL-2 therapy**




^IL-2 indicates IL-2 therapy administered

*5.2.8 Sub study phenotypic analysis*

In the nested sub-study naïve and memory cells did not change in either the CD4 or CD8 compartments. Activated CD4 and CD8 T-cells increased significantly in response to each IL-2 cycle although returned to baseline by the end of the study period (Figures 5.5 and 5.6).

**Figure 5.5: % CD4CD38+ and CD8CD38+ cells in patients receiving IL-2 therapy**

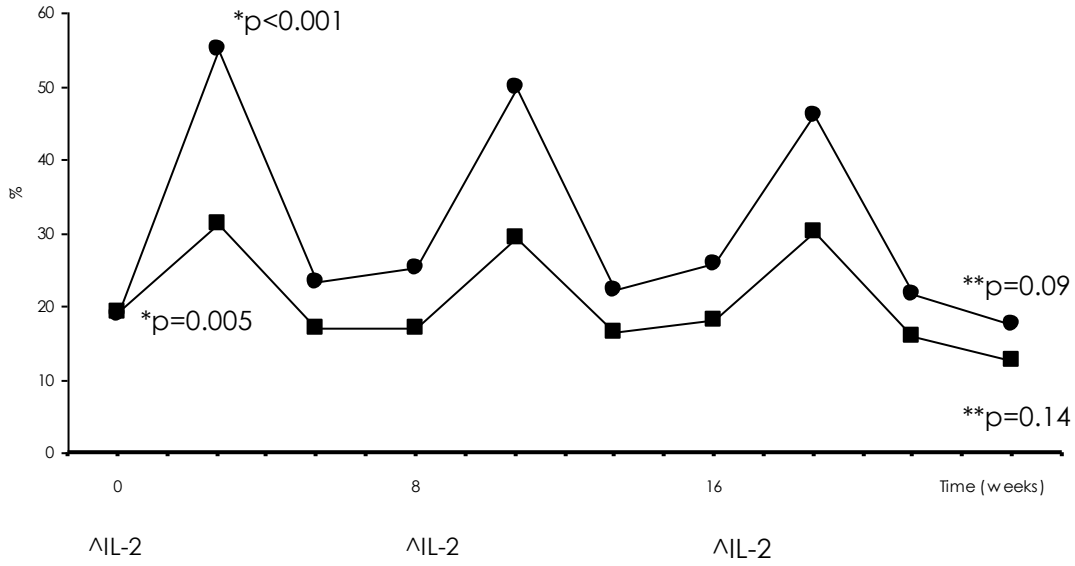
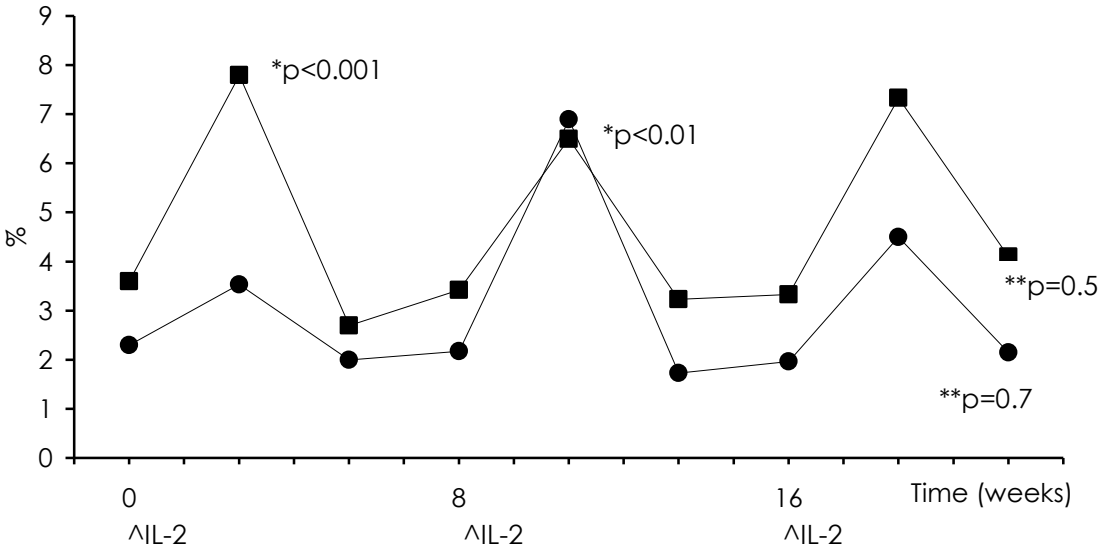



**Figure 5.6: % CD4HLA-DR+ and CD8HLA-DR+ cells in patients receiving IL-2 therapy**

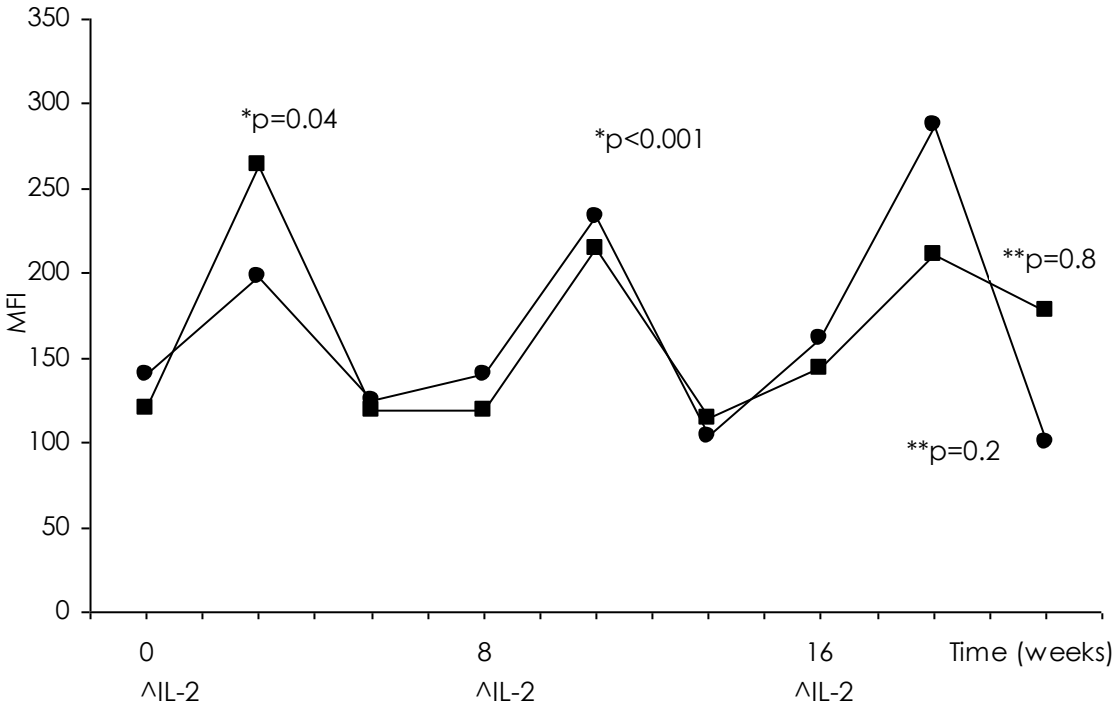



● = CD4CD38+/HLA-DR, ■ = CD8CD38+/HLA-DR, ^IL-2 indicates IL-2 therapy administered

* p comparing day 1 and day 5,** p comparing baseline and week 24

CD38 expression intensity also increased on CD8 cells in a similar pattern (Figure 5.7).

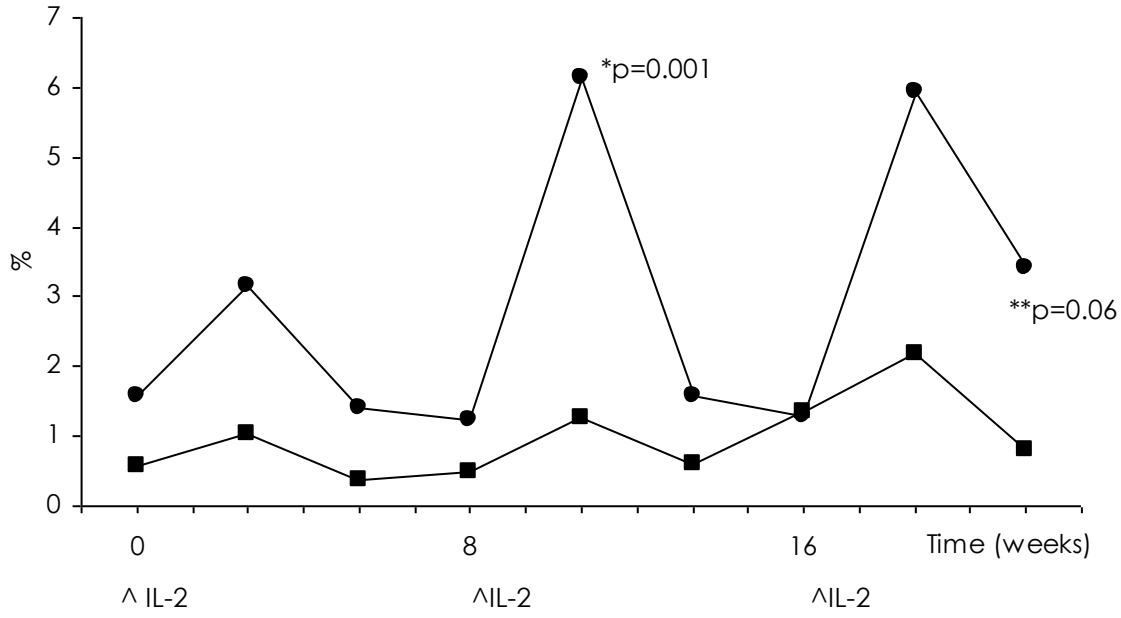
**Figure 5.7: CD38 expression on CD4 and CD8 T-cells in patients receiving IL-2 therapy**




● = CD4CD38+, ■ = CD8CD38+, ^IL-2 indicates IL-2 therapy administered
* p comparing day 1 and day 5, ** p comparing baseline and week 24

CD122 expression remained low throughout the course of the study. CD4CD28 co-receptor positive cells and CD25 receptor expression increased although did not reach significance, however the overall numbers are low (Figure 5.8). CD8+CD28+ was unchanged.

**Figure 5.8: % CD4CD25+ and CD8CD25+ cells in patients receiving IL-2 therapy**




● = CD4CD25+, ■ = CD8CD25+, ^IL-2 indicates IL-2 therapy administered
* p comparing day 1 and day 5, ** p comparing baseline and week 24

5.2.9 Toxicities and safety data

Side effects were common, as expected, and the majority were mild to moderate. No new side effects were encountered. The most frequent dose limiting side effects were constitutional signs and symptoms. Full details are provided in Chapter 8, and include flu-like symptoms, nausea and diarrhoea, injection site swelling, depressed mood and pancreatitis. Locally, one patient experienced pancreatitis post cycle 1 (7.5MIU) and again post cycle 2 (4.5 MIU), and therefore did not receive any further IL-2 therapy. Interestingly this patient also experienced marked injection site reactions and during cycle 2 those sites used for cycle 1 (different to cycle 2 injection sites) again displayed a marked though typical reaction. There were no deaths during follow-up.

*5.2.10 Protocol-defined treatment failures and clinical disease progression*

Using the protocol definition, three patients experienced treatment failure and commenced cART; two following diagnoses of KS (one control and one high dose IL-2 group) and one for a declining CD4 T-cell count (control).

*5.2.11 Discussion*

In this pilot study of intermittent IL-2 monotherapy, CD4 T-cell counts increased significantly in the IL-2 treated arm compared with the control group at week 24. Importantly, these increases were not associated with sustained increases in VL. We did observe transient bouts of viraemia on day 5 of IL-2 therapy, which was consistent with that observed in previous trials in which individuals were receiving sub-optimal cART (Kovacs *et al*., 1996, Davey *et al*., 1997, Carr *et al*., 1998) and also as described in this thesis in Chapter Four in patients on apparently suppressive cART. Despite these transient bursts, there were no long-term changes in VL. The sub-study demonstrated that, as could be predicted, activated CD4 and CD8 cells rose acutely in response to exogenous IL-2, however there was no overall rise in activated cells observed over the 24 week study period. A similar pattern was observed in the expression density of the activation markers. In addition to IL-2 cycle related increases there was an overall trend to increased CD28 and CD25 expression on CD4 T-cells. These results, although limited by the number of patients, suggest the hyperactivation associated with IL-2 is not prolonged and returns to baseline. Increased CD28 and CD25 expression could be beneficial in terms of anergy reversal with CD28 expression and an increase in Tregs (CD4+CD25+).

The clinical significance of this observed increase in CD4 T-cells produced under the influence of IL-2 therapy is uncertain and this uncertainty led to the

initiation of two large clinical end-point studies (SILCAAT and ESPRIT) to assess the clinical consequences of IL-2 in combination with antiretroviral therapy (Abrams *et al*., 2009). SILCAAT and ESPRIT are described in more detail in the Introduction (Chapter One, 1.3.2.4.4) but no clinical advantage was demonstrated with the addition of IL-2 therapy to cART. Prior to ESPRIT, four Vanguard studies were conducted to address methodological and operational issues for studies of IL-2 therapy (Losso *et al.*, 2000, Ruxrungtham *et al.*, 2000, Abrams *et al.*, 2002). This pilot study, the UK-Vanguard, was initiated to examine IL-2 treatment without ARV therapy, and in contrast to the other studies which utilized cART, showed a relatively blunted CD4 T-cell response. The mean increase in CD4 T-cell count observed at 24 weeks compared to the control arm was 132 cells/μL, compared to the other Vanguard studies with increases of 328 (Losso *et al.*, 2000) 459 (Ruxrungtham *et al.*, 2000) and 347 cells/μL blood (Abrams *et al.*, 2002). This may be due to increased destruction of newly emerging CD4 T-cells due to the ongoing viral replication, either by direct cell killing or via increased levels of immune system activation. As similar IL-2 doses were administered in all the Vanguard studies it is unlikely to be an IL-2 dose related effect. Although IL-2 in combination with antiretroviral therapy may yield more robust immunologic responses at 24 weeks, IL-2 monotherapy warrants further investigation including clinical outcome trials, especially in antiretroviral treatment naïve patients who wish to maintain a CD4 T-cell count above a level at which cART would be indicated. The other scenario in which IL-2 therapy may be effective is in structured treatment interruptions (STI) and treatment cessation to maintain the CD T-cell count at a level sufficient to avoid the need to restart treatment. These approaches have recently lost favour, largely due to concerns regarding emerging resistance and adverse clinical outcomes as

seen in the SMART study (El-Sadr *et al.,* 2006), which was possibly related to immune system re-activation. However alternative strategies utilizing IL-2 therapy may be worth exploring.

As observed in other trials, this study demonstrates that IL-2 is well tolerated at doses that produce significant increases in CD4 T-cell counts (Miller *et al*., 2001). Toxicities occurred only during the intermittent cycles of IL-2, were mild to moderate in severity and were managed with a comprehensive approach that included dose modification, prophylactic medications, and withholding IL-2 in only one case (pancreatitis). No novel toxicities were observed.

While the sample size for this study was only 36, the results indicate that at least modest CD4 T-cell increases are possible without adversely affecting viral load, and further studies are warranted to examine IL-2's potential for increasing or maintaining CD4 T cell counts and thereby delaying the need to initiate cART. Given the results of SLICAAT and ESPRIT it will be important to include clinical outcome measures in these studies.

In summary, this study demonstrated that IL-2 monotherapy significantly increased CD4 T-cell counts without adversely effecting the VL or markers of immune activation. Although the clinical effect of the rise in co-receptor and IL-2 receptor expression is not known, in this treatment setting these are novel observations and warrant further investigation.

## 5.3 Interleukin-2 therapy in HIV-1 infected individuals with late stage disease and non-suppressive antiretroviral therapy

### *5.3.1 Background*

There are a number of HIV-1 infected individuals who are heavily ARV experienced on non-suppressive regimens with limited treatment options due to treatment side effects or viral resistance; often a result of prior sequential mono/dual therapy. In some instances a potentially effective regimen can be constructed but may be awaiting commencement of an expanded access programme (EAP), drug licensing or investigation results such as viral resistance or tropism. Such individuals often have very low CD4 T-cell counts and are at significant risk of disease progression during this period. Such a situation existed in our clinic and it was decided to offer this small number of affected patients IL-2 in a 'compassionate release' programme, as these individuals did not qualify for the IL-2 studies enrolling in our service at that time. The second part of this chapter is a description of the findings of this programme.

### *5.3.2 Treatment*

HIV-1 infected patients with very low CD4 T-cell numbers (<50 cells/μL) and virological failure on what was considered at the time to be potentially viral disabling ARV regimens while awaiting EAP were offered IL-2 therapy. IL-2 therapy consisted of 5 MU twice daily subcutaneously for 5 days given at 4 week intervals for three cycles. Prophylactic medication to limit IL-2 side effects was prescribed. CD4 T-cell counts and HIV-1 viral loads were monitored at baseline, day one of each cycle and three weeks after completion of the final course of IL-2. Side effects and adverse events were recorded and routine safety bloods were performed for each cycle.

*5.3.3 Results*

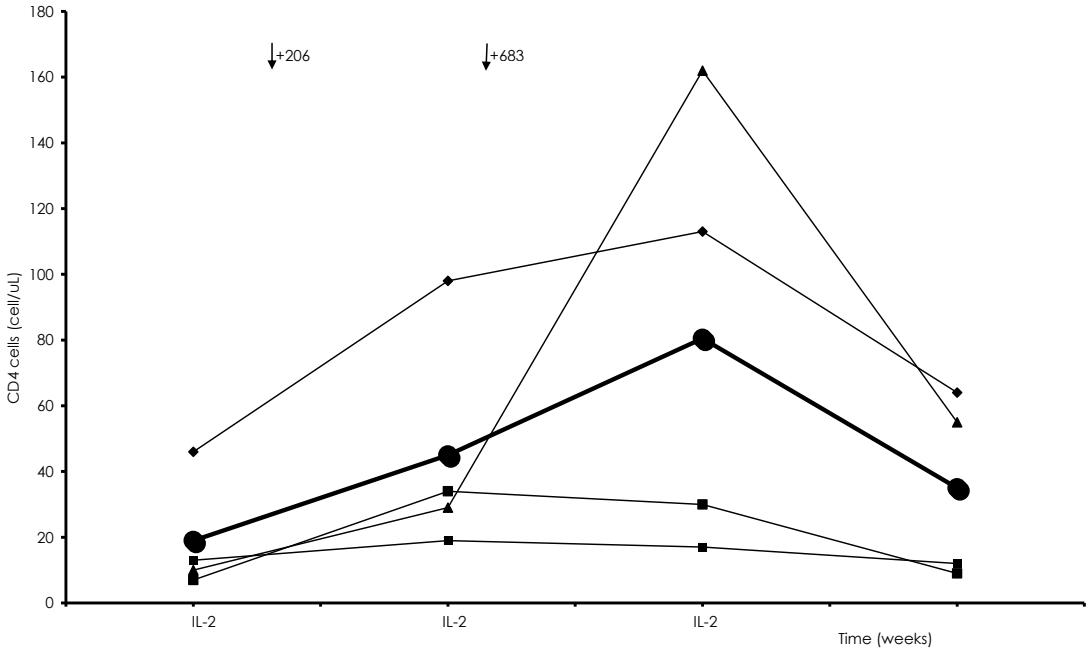
Four patients accessed IL-2 therapy, all were MSM with a mean age of 37.25 years (range 33-45). All had previous AIDS defining diagnoses. Three were taking Nelfinavir and 3TC and one Saquinavir with 3TC. The mean treatment period of their current regimen was 6 months (range 4-7). The mean baseline CD4 T-cell count was 19 cells/μL (range 7-46), %CD4 was 4% (range 2-8) and VL 270,853 copies/ml (range 47,730-500,000). The four patients received 11 cycles of IL-2. All patients experienced flu-like symptoms (one severe, requiring dose reduction and delayed cycle 3 and one moderate requiring dose reduction for cycle 3). There were no other serious side effects and no other dose limiting toxicities.

*5.3.4 IL-2 therapy response*

During therapy two patients responded well with CD4 T-cell counts >100 cells/μl, one patient's CD4 T cell count quadrupled and one patient showed no CD4 T cell count response.

The mean peak CD4 T-cell count occurring at any time during IL-2 therapy was 236 cells/μl blood (range 19-683) and %CD4 was 15% (range 2-26). Four weeks after the initiation of each IL-2 cycle the mean CD4 T-cell count for all cycles was 47 cells/μl (8%). At follow-up the mean CD4 cell count (%) was 35 cells/μL (6%) (Figures 5.9 and 5.10) and VL was 409,448 copies/ml. The mean NK cells and percentage rose in response to the IL-2 cycles, again some individuals showed a greater response and levels after three cycles were comparable to baseline (Figures 5.11 and 5.12).

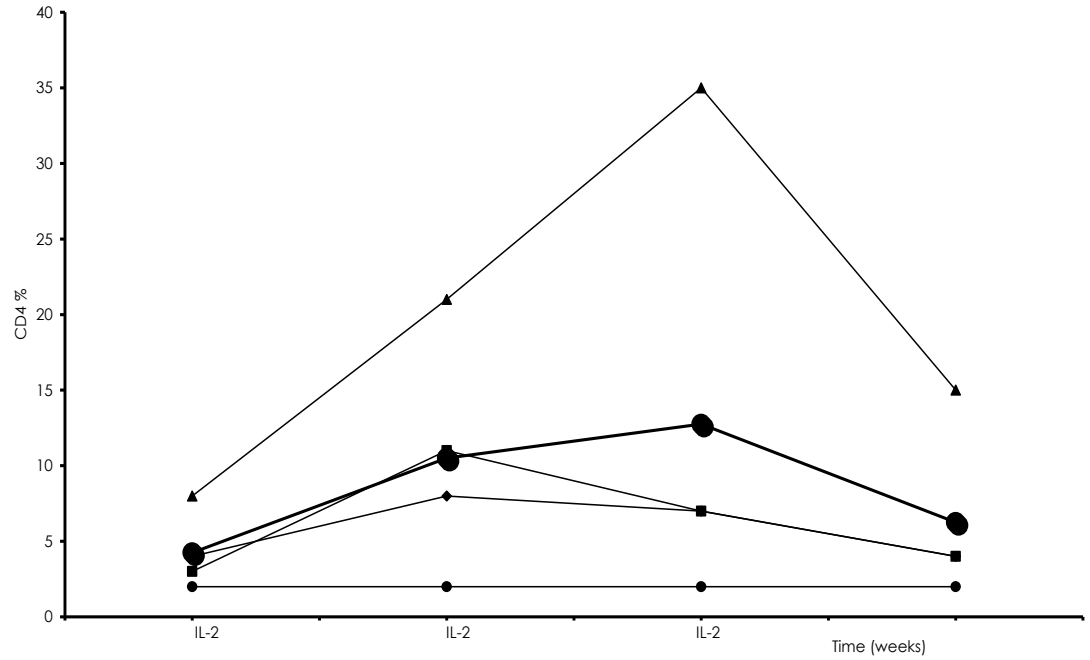
**Figure 5.9: Individual and mean CD4 T-cell counts**




Patient 1 = ♦, patient 2 = ■, patient 3 = ▲, patient 4 = ●, mean = ●

IL-2 indicates IL-2 therapy administered

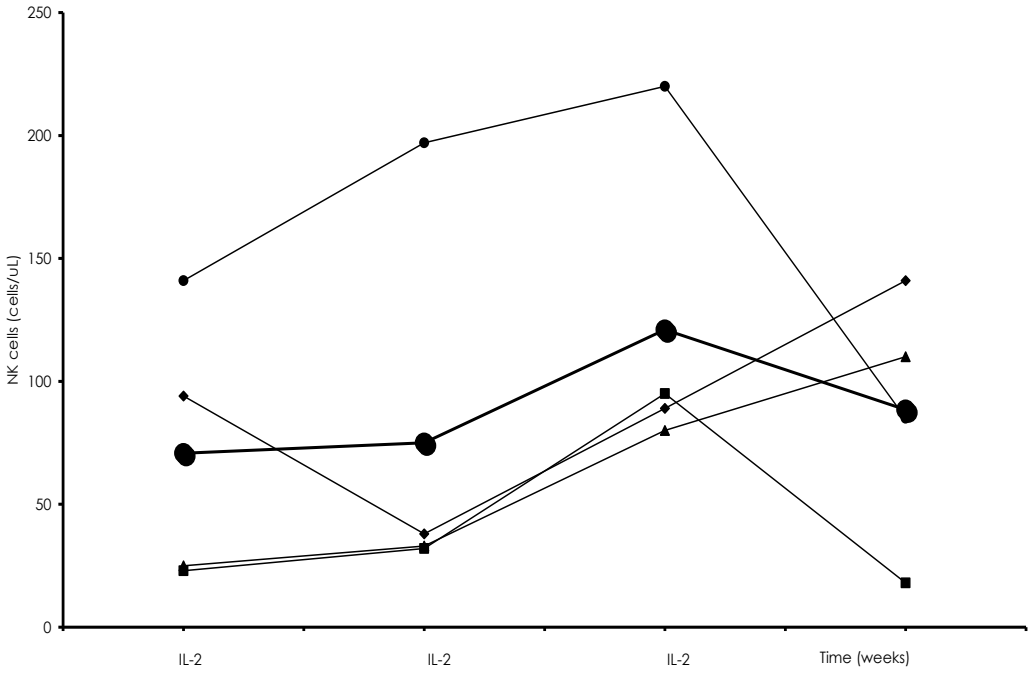
**Figure5.10: Individual and mean CD4 T-cell percentages**




Patient 1 = ♦, patient 2 = ■, patient 3 = ▲, patient 4 = ●, mean = ●

IL-2 indicates IL-2 therapy administered

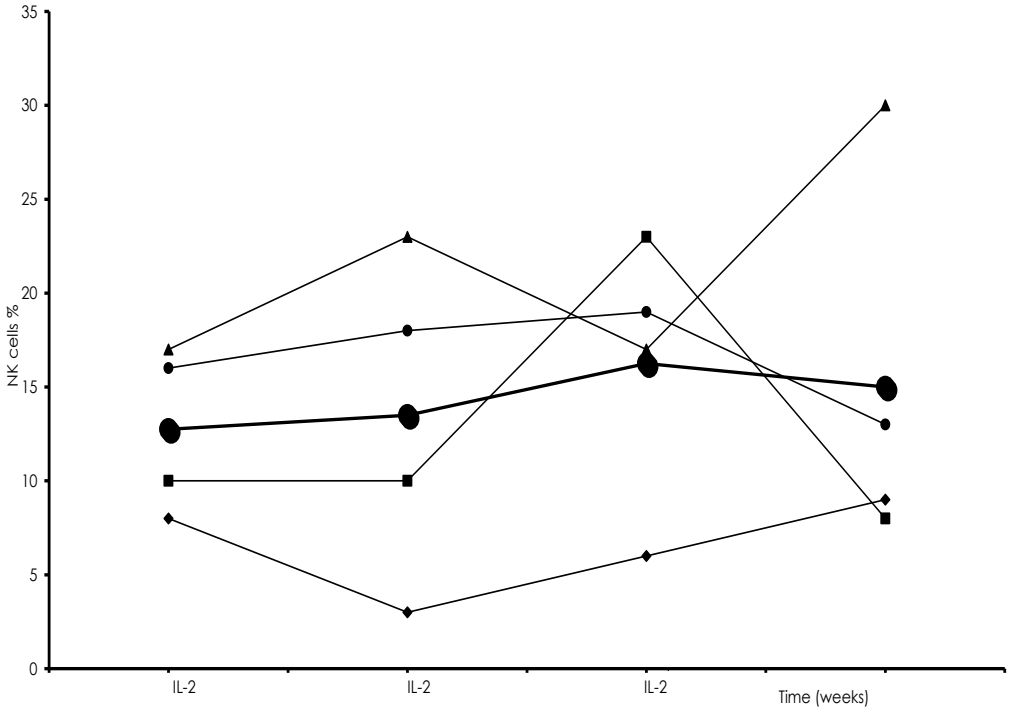
**Figure 5.11: Individual and mean NK cell counts**




Patient 1 = ♦, patient 2 = ■, patient 3 = ▲, patient 4 = ●, mean = ●

IL-2 indicates IL-2 therapy administered

**Figure 5.12: Individual and mean NK cell percentages**




Patient 1 = ♦, patient 2 = ■, patient 3 = ▲, patient 4 = ●, mean = ●

IL-2 indicates IL-2 therapy administered

*5.3.5 Discussion*

In this small group of patients IL-2 therapy caused no serious side effects and no clinical events occurred during the treatment period. All patients subsequently entered the expanded access programme (EAP). It may be that patients with advanced stage disease require a greater number of cycles or ongoing IL-2 therapy to achieve a sustained beneficial effect. Additionally the rise in CD4 T-cell count in response to the IL-2 therapy was in the context of non-suppressive ARV regimens, and therefore the rise was not sustained. In this small study it would appear IL-2 was an effective therapeutic intervention in some patients while awaiting EAP and warrants further investigation and possible consideration in patients with very advanced disease. There did appear to be a differential effect whereby some patients responded with marked rises in SIM and some did not, again highlighting the potential role of genetic and virologial influences on immune therapy. Furthermore the significant initial rise seen in two patients may reflect redistribution rather than *de novo* production of T-cells. Clearly the numbers are too small to make definitive conclusions but the findings warrant further investigation. In such cases IL-2 on its own might not be enough and approaches combining IL-2 with other biological agents (including other cytokines) that display distinct effects and target distinct components of the immune response may be more effective.

## CHAPTER SIX: DISCUSSION AND CONCLUSIONS

The introduction and subsequent refinement of antiretroviral therapy for HIV-1 infection has undoubtedly revolutionised the care of individuals infected with HIV-1 worldwide. Through viral suppression it allows immune recovery, both quantitative and qualitative (Kelleher *et a*l.1996, Autran *et al.,* 1997, Staszewski S *et al.,* 1999, Pakker *et al*., 1998, Gallant JE *et al*., 2006, Mills AM *et al.,* 2009, Molina JM *et al*., 2010). However this restoration is not complete and there is evidence that once lost, some facets of immune function cannot be restored utilising current therapeutic strategies (Autran *et al*., 1997, Hardy *et al*., 1999). In my thesis I have endeavoured to explore the clinical outcomes of cART, examining both its beneficial effects and residual deficiencies, and thereby set the scene for a more in-depth exploration of immunological therapies which may address such therapeutic inadequacies.

The mortality audit and discordant CD4 response study (Chapter Three) demonstrate significant benefit from cART; they also highlight the ongoing issue of incomplete qualitative immune recovery on cART. Although mortality has declined dramatically with the introduction of effective ARVT (Pallela *et al.*, 1998, Lederman *et al.,* 2000) and many patients dying with HIV-1 in the UK do so due to late diagnosis and delayed treatment initiation (Lucas *et al*., 2008), there remains a significant number who are still dying from conditions, such as lymphoma and other cancers, related to incomplete immune recovery despite fully suppressive cART (Lewden *et al*., 2007). This audit collected data on a large number of patients, attending two different UK centres over a long period. It demonstrated a fall in the crude all cause mortality rate from 7.3 to 2.6%, and a rise in the median CD4 T-cell count at death from 20 to 116 cells/μL blood. As with all retrospective audits however

it has its limitations, mainly related to data capture through inadequate documentation. I addressed these concerns by widening the data sources to include physician interviews, hospice and other hospitals' records and coroner's records as well as our local hospital records. Whilst I believe this maximised the accuracy regarding causes of death, the details of cART, in particular previous combination history and reasons for therapy change and cessation is less robust.

The discordant CD4 response cohort data demonstrates that not every individual realises the ideal treatment response. The most pertinent finding within this study is that an adequate CD4 T-cell rise is a clear predictor in terms of clinical outcome, irrespective of virological suppression. The findings add support to the theory that ongoing immune activation plays a role in those patients experiencing a discordant immunological response; with inadequate CD4 T-cell recovery despite an apparently fully suppressed viral load (Garcia *et al.*, 2000, Leng *et al*., 2001, Anthony *et al.*, 2003). This may be due to several factors. There may be ongoing low level viral replication which is not captured with routine VL monitoring due to current test sensitivity and the timing of sampling. Viral production from latently infected cells may be causing low level immune activation with the associated sequelae and reseeding of resting T-cells (Kelleher, 2008). Additionally antigenic stimulation from translocation of foreign proteins across the gut mucosa may play a key role in the generalised immune activation, with HIV-1 in fact playing more of a bystander role (Douek *et al.,* 2009). It is thought that GALT is significantly depleted relatively early in HIV-1 infection (Brenchley *et al*., 2004), and this permits the passage of foreign proteins resulting in immune hyperactivation (Brenchley *et al*., 2006). Hyperactivation as measured by CD8+CD38+ is a prognostic indicator (Liu *et al*., 1997, Douek *et al*., 2009), and could

potentially be used as an extra SIM in addition to the CD4 T-cell count to predict treatment response, and also possibly to identify patients who may be suitable for additional therapeutic interventions; whether this be intensification of cART or immune therapies such as IL-2, IL-7 (Sereti *et al*., 2009) or immunosuppressants such as hydroxychloroquine (Richman *et al.,* 2009). It is of interest that further support is afforded within the IL-2 Remune study where those patients who failed to achieve the CD4 T-cell requirement of 300 cells/μL blood for randomisation had higher mean baseline CD8+CD38+ T-cells and markedly less decline over the 16 weeks of cART with higher week 16 levels of activation as measured by this SIM. We decided this cut-off on the basis of other work suggesting better IL-2 responses in those with higher CD4 T-cell counts (Davey *et al,* 1997), and we may therefore have inadvertently excluded the very patients who may have demonstrated benefit. This adds further support to the use of additional or alternative SIM in initiating and monitoring immune therapy. The association I describe between the slope of the pre-cART CD4 T-cell decline and subsequent CD4 T-cell rise, not previously reported, could be used to produce a model for predicting cART outcome, and may even be of use in deciding the initial cART regimen or early interventions to address inadequate recovery. The effect on clinical outcomes such as disease progression and death of an adequate rise in the CD4 T-cell count regardless of the cART mediated effect on VL suggests measures which increase CD4 T-cell counts, possibly regardless of the effect on VL, may prove to be beneficial. This study included over one thousand patients recruited over six years and followed over a two year period, however as a retrospective cohort study it has inherent limitations, including potential bias and missing data. The statistical method employed (Chapter Three, 3.3.3) was chosen to reduce these

limitations as much as possible within this study design. The fact that all patients attended one treatment centre may affect some of these findings being applied to other settings. Although National prescribing guidelines are adhered to at this centre, individual physicians may respond differently to the different treatment outcome scenarios, and within different time frames. The other main limitation of this and other similar studies (Picketty *et al*., 1998, Perrin *et al*., 1998, Barreiro *et al*., 1999, Grabar *et al*., 2000) is a lack of uniform definitions. Additionally duration of follow-up and the inclusion of non-ART naïve patients in other studies make cross study comparisons difficult. However I believe given a difference was observed in clinical outcomes using our relatively generous definitions and that this difference was observed at 12 months, supports the definitions and time periods I selected. Building on these findings, possible future studies would clearly include longer term follow-up of this particular cohort, as well as studies to explore the potential of other SIM both as predictive and monitoring measures. Intervention studies would explore whether cART manipulation and/or immune therapies may influence discordant responses and whether this translates to clinical benefit.

The above study did not demonstrate any difference between the two classes of ARVT when only boosted PI therapy (now standard of care) was considered. There is however some *in vitro* data of a differential effect of NNRTI and PI on immune cell function (Andre *et al.*, 1998, Chavan *et al.*, 2001). The aim of the next study (Chapter Three, section 3.4) therefore was to see if a switch in therapy, already planned for a clinical indication, impacted on the individual's HIV-1 specific responses, as well as recall antigen responses and IL-2 responsiveness. We did find an improvement in all three parameters including HIV-1 specific responses following a switch to a NNRTI

based regimen. Unlike similar recovery in other studies (Gotch *et al.*, 1999) the responses were sustained out to 24 weeks. Although HIV-1 specific responses are recognised as advantageous in, for example LTNP and elite controllers (Westrop *et al.,* 2009), it is not known whether their restoration translates to clinical advantage. Although intuitively one would assume this to be the case it would need to be demonstrated before it could be advocated as a therapeutic strategy (Imami *et al*., 2007). Demonstrating this in a larger cohort and over a prolonged period would be a significant undertaking and pragmatically of unclear benefit as NNRTIs are currently the first line recommended ARVT in the UK (Gazzard *et al*., 2008). A smaller more realistic study would be to examine any potential association between this possible recovery of HIV-1 specific responses with improved control of low level viral replication and production and resultant improvement in immune activation markers. If these findings are borne out in larger studies, it may well have implications for future trials of immune therapy, and potentially the findings of IL-2 studies to date.

On this background I then carried out several further studies to examine in more detail the effect of cART, IL-2 and Remune on the immune system in different therapeutic settings. The aims of this work were to examine whether, in addition to confirming the findings of other researchers in respect to IL-2 and standard SIM, we could identify any new effects, particularly in relation to the novel immune therapeutic strategy we employed in the IL-2 Remune study. I was also interested to see if we could define alternative or additional SIM to drive IL-2 scheduling and monitor its effect. Furthermore I wanted to explore possible benefits in those on no cART and in those with few treatment options at extreme risk of clinical progression. In summary, the studies found a beneficial effect on several parameters which translate to a degree of

immune recovery. The side effects were tolerable for the majority of patients. Given the fact that these were all pilot studies or compassionate release programmes, it is not possible to draw firm conclusions due to the small number of patients with resulting wide confidence intervals, however several interesting findings were observed. IL-2 causes a return towards normalisation of absolute CD4 and CD8 T-cells and their ratio in the majority of patients. We also observed a decrease in T-cell activation in patients who received IL-2. These changes were of a greater magnitude and more sustained in those patients receiving Remune in addition to IL-2. The rise in CD4 T-cells would be expected to translate to clinical benefit but in the large clinical outcome trials to date (Abrams *et al*., 2009), this does not appear to be the case. There are several possible explanations for this. It may be the CD4 T-cells associated with IL-2 therapy are in some way different to those associated with cART and do not confer benefit, as suggested by Sereti and colleagues (Sereti *et a*l., 2000). IL-2 may induce some other effect which we are not capturing which negates the potential benefit of the elevated CD4 T-cell count. It may also be that we are using the incorrect SIM to drive repeated cycles and are therefore not maximising the potential therapeutic benefit of IL-2 therapy. Patient selection may have reduced any potential beneficial effects of IL-2 therapy by not mandating a high CD4 cell nadir and effective virological control. It may also be that IL-2 therapy should be more targeted to certain patients who may benefit more, due to specific characteristics, for example ongoing immune activation or a discordant immune response in relation to CD4 T-cell recovery. A novel finding in the Remune IL2 study of particular interest is the viral load blips associated with the IL-2 cycles which resulted in recovery of HIV-1 specific responses, some of which were sustained. This is in contrast to some of the STI studies and suggests a

synergistc action between IL-2 and low level viraemia. Despite associated IL-2 cycle related activation, this did not result in a sustained hyperactivation, and in fact there was less activation of CD8 T-cells in those patients who had received IL-2 and Remune. We also observed significant increases in CD25 expression. This surface receptor is a marker for both activated T cells and Tregs; cells which play a key role in immunomodulation and control of the immune response. One limitation of this observation was that we did not include other markers to better characterise these cells which were not available at the commencement of this study. Some authors believe IL-2 induced Tregs are less immunosuppressive than those stimulated by 'natural' antigen (Sereti *et al.,* 2000). However we did not find evidence to support this overall, as in the IL-2 Remune study we observed both a sustained increase in CD4+CD25+ T-cells and a reduction in immune activation. However it may be this lack of suppressive function is reflected in the peri-cycle increase in activation and HIV-1 specific responses; not being suppressed by the cycle associated rise in Tregs. The subsequent stimulation with Remune may then have influenced these cells to behave in a more typical fashion. This however is highly speculative and these observations can only be considered an association not causation. The observations however are of sufficient interest and novelty to warrant further investigation. In several analyses it did appear the observed differences between the two groups were being driven by the patients who received both IL-2 and Remune and in some instances the responses were more sustained in this arm. This was observed as described above for CD4 and CD8 T-cell counts and CD8 activation and also for the co-receptor CD28 expression. The Remune was administered with the first IL-2 cycle and then at three time points subsequent to the final cycle of IL-2 and it may be there is a synergistic

effect, with IL-2 priming the immune system to be more receptive to therapeutic immunisation. It is of interest to note the change in CD4 T-cells was more marked and sustained in the memory/effector subset. The lack of any real difference in DTH as measured clinically again raises the question of translation to clinical benefit. However DTH is a relatively insensitive measure and the scheduling we employed for IL-2 and Remune administration may have influenced this to a degree.

These studies were exploratory pilot studies and a compassionate release programme and as such were not powered to detect a significant difference between the groups. However the IL-2 Remune study was a randomised controlled study and this should address some of the potential biases. As with all IL-2 studies the side effect profile of IL-2 does not permit placebo arms. Although clearly limited by the small numbers the novel approach to the administration of immune therapy resulted in several novel, interesting observations and raises several questions which should be further explored in larger studies (Downey *et al.*, 2010).

In conclusion, cART contributes significantly to immune reconstitution but it is incomplete. IL-2 and therapeutic immunisation result in enhancement of the immune recovery seen with cART, at least at a cellular level. The challenge is to determine how this can be translated to clinical benefit. Using additional SIM, such as CD38, CD28 and CD25, may help in determining those who may benefit most, as well as monitoring treatment response and driving maintenance therapy. The timing and schedule employed will be key and warrants further investigation. It is likely however, as with ARVT, immunotherapy will require a similar approach with a combination of different biological therapies acting via various different mechanisms and targeting distinct components of the immune system to produce a variety of

changes to better normalise immune function. This could potentially consist of cytokines (for *e.g.* IL-2, IL-7, GM-CSF), therapeutic immunisation, ideally inducing both HIV specific responses and neutralising antibodies, and immuno-modulators such as hydroxychloroquine. Further research is required and although demonstrating clinical benefit is essential to underpin any therapeutic recommendation, the recent negative clinical trial results should not lead to the abandonment of IL-2 as a potential HIV -1 therapy; it may be that similar to zidovudine being used as monotherapy in the eighties, we haven't got it wrong; we just haven't quite got it right.

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## CHAPTER EIGHT WORK ACCEPTED FOR PUBLICATION AND PRESENTATIONS

The first section relates to work included in the thesis; published papers are in Appendix. The second section is additional research I have carried out in collaboration with colleagues as part of the studies included in my thesis.

### Section One

Papers and letters published in peer review journals

Sullivan AK, Burton CT, Nelson MR, Moyle G, Mandalia S, Gotch F, Gazzard BG, Imami N. Restoration of HIV-1 specific responses in HIV-1 infected individuals changing from protease to non-nucleoside reverse transcriptase inhibitor containing antiretroviral therapy. *Scand J Immun* 2003; 57:600-7

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Imami N, Sullivan AK, Gotch FM. Immunomodulation in HIV-1 infection in the HAART era. *J HIV Therapy* 2001; 6:77-84

Gotch F, Burton C, Donaghy H, Hardy G, Imami N, Pido-Lopez J, Pires A, Sullivan A. Novel approaches to the treatment of HIV-1. *HIV & AIDS Current trends* 2001; 7:7-8

Peer reviewed abstract presentations

Sullivan AK, Mandalia S, Nelson MR, Gazzard BG. Discordant immunological and virological responses to HAART in ARV naïve HIV infected individuals. *IAS,* Rio de Janeiro, July 2005, WePe12.2CO1

Tung MY, Sullivan AK, Mandalia S, Nelson MR, Gazzard BG. Discordant responses to HAART in ARV naïve HIV infected individuals. *HIV Medicine* 2005; 6:5

Sullivan AK, Hardy G, Burton C, Pires A, Nelson M, Gotch G, Gazzard B, Imami N. Effect of IL-2 therapy on T-cell phenotype, activation and IL-2 receptor expression. *10th CROI*, Boston February 2003 [abstract 651]

1. Sullivan AK, Amjadi P, Nelson MR, Tavel J, Gotch FM, Youle M, Gazzard BG. Interleukin-2 therapy in HIV-1 infected ARV naive patients: effect on T cell activation. [abstract 109] oral presentation
2. Sullivan AK, Imami N, Hardy G, Nelson MR, Moss R, Gotch FM, Gazzard BG. Viral blips and HIV-1 specific helper T cell responses in patients on HAART receiving IL-2 therapy with or without a therapeutic vaccine. [abstract 660]
1st *IAS Conference on HIV pathogenesis and treatment*, Buenos Aires July 2001

1. Sullivan AK, Imami N, Hardy G, Burton C, Pires A, Nelson MR, Moss R, Gotch FM, Gazzard BG. HIV-1 specific lymphoproliferative responses and viral blips in patients on HAART receiving IL-2 therapy with or without Remune. [abstract O26] oral presentation
2. Sullivan AK, Amjadi P, Richardson C, Nelson MR, Tavel J, Gotch FM, Youle M, Gazzard BG. Effect of interleukin-2 therapy on T cell phenotypes in HIV-1 infected patients receiving no antiretroviral therapy. [abstract O36] oral presentation
*7th Annual Meeting of the British HIV Association*, Brighton April 2001

1. Sullivan AK, Imami N, Hardy GAD, Pires A, O'Donavan B, Nelson MR, Gotch FM, Gazzard BG. Acute viral load blips in response to subcutaneous interleukin-2 therapy in HIV-1 infected individuals on highly active antiretroviral therapy. [abstract P199]
2. Sullivan AK, Pires A, Hardy GAD, Gotch FM, Gazzard BG, Imami N. Phenotypic T cell changes in HIV-1 infected individuals receiving highly active antiretroviral therapy. [abstract P204]
3. Sullivan AK, Nelson MR, Pozniak AL, Gazzard BG. Interleukin-2 therapy in HIV-1 infected individuals with late stage disease and failing antiretroviral therapy. [abstract P200]
*5th International Congress on Drug therapy in HIV Infection*, Glasgow October 2000

1. Sullivan AK, Nelson MR, Mandhalia S, Moyle G, Gotch F, Gazzard BG, Imami N. Immune responses in patients changing from PI to NNRTI based HAART. [abstract WePe4189]
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1. Sullivan AK, Mazhude C, Nelson M, Fisher M, Chard S, Gazzard B. Mortality in the HAART era. [abstract O24] oral presentation
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*6th Annual Meeting of the British HIV Association*, Edinburgh March 2000

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Sullivan AK, Vlahakis E, Mandalia S, Nelson M, Gazzard B. Discordant CD4 response on HAART. *7th European Conference on Clinical Aspects and Treatment of HIV Infection,* Lisbon October 1999 [abstract 219] oral presentation

**Section Two**
Hardy G, Imami N, Nelson M, Sullivan AK, Moss R, Aasa-Chapman M, Gazzard B, Gotch F. A Phase I, Randomized Study of Combined IL-2 and Therapeutic Vaccination with Anti Retroviral Therapy. *J Immune Based Ther Vaccines* 2007; 11:5:6

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Hardy GAD, Imami N, Sullivan AK, Nelson MR, Burton CT, Gazzard BG, Gotch FM. Reconstitution of CD4 T cell responses in HIV-1 infected individuals initiating HAART is associated with renewed IL-2 production Responsiveness. *Clin Exp Immun* 2003; 134:98-106

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2. Pido-Lopez J, Imami N, Pires A, Sullivan A, Burton C, Hardy G, Gazzard B, Aspinall R, Gotch F. Limited thymic contribution to CD4 Tcell restoration during early HAART.
*8th CROI*, Chicago USA, Feb 2001

Burton C, Sullivan A, Imami N. Assessment of chemokines in HIV-1 infected individuals: impact of PI vs NNRTI based HAART. *13th Int AIDS Conference,* Durban July 2000

1. Hardy GAD, Imami N, Sullivan A, Burton CT, Nelson N, Gazzard B, Gotch FM. T-lymphocyte function in HIV-1 infection: effects of HAART.
2. G Hardy, N Imami, A Sullivan, J Wilson, C Burton, R Moss, B Gazzard, F Gotch. Effects of combined treatment with IL-2 and an inactivated gp120 depleted HIV-1 immunogen (remune) on immune reconstitution in HAART treated HIV-1 infected individuals.
*BSI/BSACI Congress*, 1999

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# Interleukin-2-associated viral breakthroughs induce HIV-1-specific CD4 T cell responses in patients on fully suppressive highly active antiretroviral therapy

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The combination of intermittent subcutaneous IL-2 and highly active antiretroviral therapy in individuals infected with HIV-1 has been shown to have a beneficial quantitative effect on the CD4 T cell count. We observed IL-2-associated viral load 'blips' inducing HIV-1-specific lymphoproliferative responses at 24 weeks in such individuals. This immunotherapeutic approach, utilizing autologous virus as autovaccination, may be a viable, safer alternative to structured treatment interruption and potentially more efficacious than therapeutic vaccines.
Intermittent subcutaneous low-dose IL-2 in combination with highly active antiretroviral therapy (HAART) has been shown to increase CD4 T cell counts at all stages of disease [1,2]. It appears, however, that the eradication of virus is unlikely unless additional immune strategies can be employed both to target latently infected cells and to enable qualitative HIV-1-specific immune reconstitution [3]. Animal models suggest that the provision of antigenic stimulation will be essential to this endeavour [4]. Treatment interruptions and therapeutic immunizations are currently being explored; the former having inherent risks of uncontrolled viral rebound, CD4 T cell loss, the reseeding of viral reservoirs and drug resistance. To date little success has been achieved in chronic infection with either strategy.
Fourteen patients were enrolled to assess the acute effect of IL-2 on the plasma viral load and HIV-1-specific responses. Patients gave written informed consent and were commenced on HAART 16 weeks before IL-2 therapy when a viral load below the level of detection (50 copies/ml) was required. IL-2 was administered at a dose of $5 \times 10^6$ units subcutaneously, twice a day for 5 days, for three 4-weekly cycles. Samples were obtained on days 1 and 5 of each IL-2 cycle for the CD4 T cell count and viral load, and at weeks 0, 4, 8, 12 and 24 for the CD4 T cell count, viral load and lymphoproliferative responses to HIV-1 recombinant antigens (nef, gp120 and p24) [5]. Results are expressed as a stimulation index (SI) in which a positive result is an SI of 5 or greater.
All 14 patients received a total of 42 cycles of IL-2. Nine of the 14 patients had at least one day 5 viral load 'blip', with a total of 13 blips (range 51-355 copies/ml). The mean viral load increased for all IL-2 cycles, day 1 versus day 5: 50 versus 91 copies/ml ($P$ = 0.002) (Fig. 1a). The mean CD4 T cell count increased from 430 to 1073 cells/μl ($P$ = 0.001). The mean SI for HIV-1-specific antigens increased above 5 at week 24 (Fig. 1b). The proportion of patients with a positive SI to any HIV-1

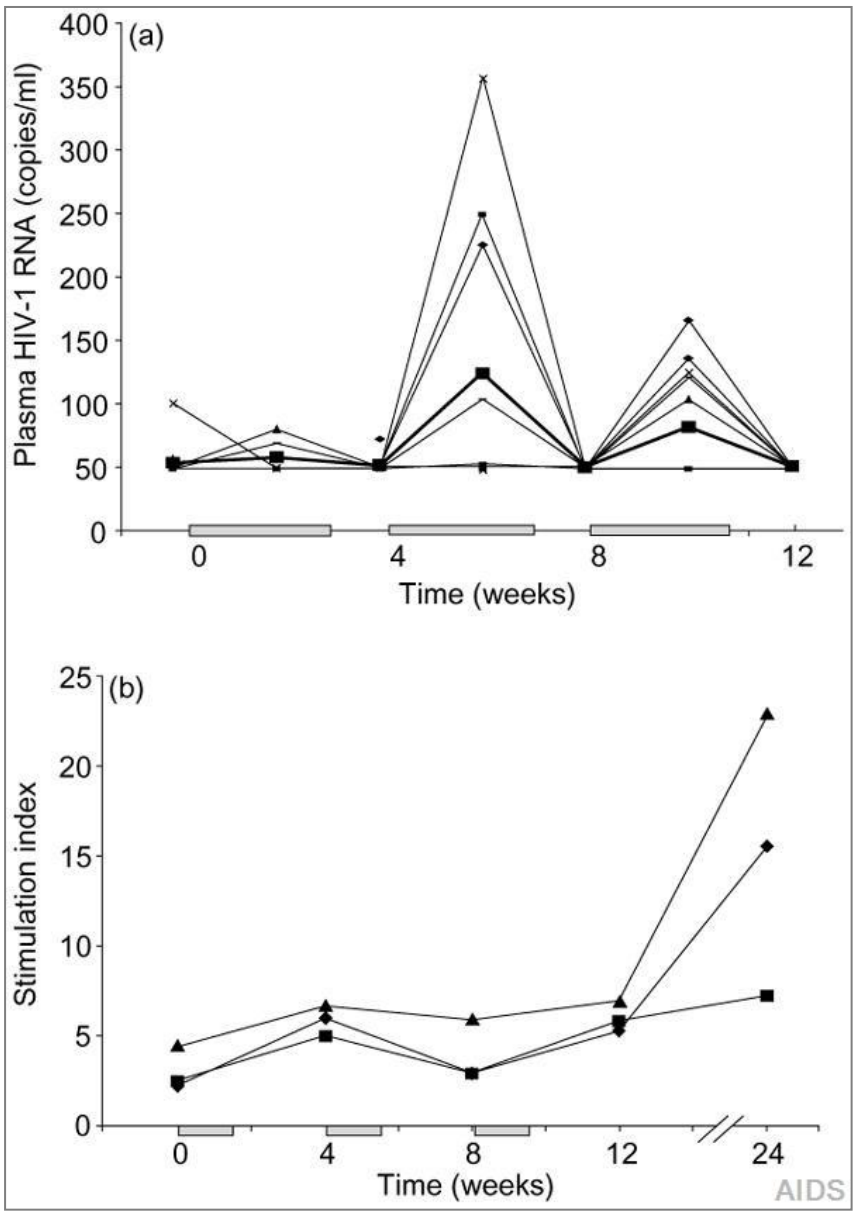


Fig. 1.Acute effect of IL-2 on the plasma viral load and HIV-1-specific responses of 14 patients enrolled in the study. (a) Plasma HIV-1-RNA load for individual patients. ▪, Mean. (b) Mean HIV-1-specific responses. ◇, nef; ▪, gp120; ▲ p24; ▒, IL-2 therapy.

For the first time we have shown that low-dose intermittent IL-2 induces viral breakthroughs in the context of fully suppressive HAART in chronic HIV infection. These blips are controlled, but are sufficient to induce HIV-1-specific responses, which are maintained for 24 weeks. This suggests a possible role for IL-2 as a safe method of autoimmunization in contrast to treatment interruption; providing autologous virus in the context of HAART while preventing significant viral rebound, protecting CD4 T cells, preventing the reseeding of reservoirs, and purging latently infected cells. Several major questions remain unanswered: the detailed dynamics and possible minimum threshold required of IL-2-associated viral blips and the duration of the induced HIV-1-specific responses. Answers should enable the creation of

immunotherapy treatment schedules that will augment the advances seen in HIV care since the introduction of HAART.

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